U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Biogeografia e diversificação de um grupo de anuros Neotropicais, *Phyllomedusa burmeisteri*

Abordagem integrativa através de análises moleculares e modelos de nicho ecológico

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Nota Prévia

Na elaboração desta tese, e nos termos do número 2 do Artigo 4o do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto e do Artigo 31º do D.L. 74/2006, de 24 de Março, com a nova redação introduzida pelo D.L. 230/2009, de 14 de Setembro, foi efetuado o aproveitamento total de um conjunto coerente de trabalhos de investigação já publicados ou submetidos para publicação em revistas internacionais indexadas e com arbitragem científica, os quais integram alguns dos capítulos da presente tese. Tendo em conta que os referidos trabalhos foram realizados com a colaboração de outros autores, o candidato esclarece que, em todos eles, participou ativamente na sua conceção, na obtenção, análise e discussão de resultados, bem como na elaboração da sua forma publicada.

À minha família,
e
à conexão Brasil-Portugal

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Será que, à medida que você vai vivendo,
andando, viajando,
vai ficando cada vez mais estrangeiro?
Deve haver um porto.

— Caio Fernando de Abreu

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Sumário

Os padrões de diversificação biológica de regiões de elevada biodiversidade, como as Tropicais e as Neotropicais, têm vindo a ser alvo de um crescente número de estudos. Trabalhos filogeográficos recentes de organismos endémicos da Mata Atlântica (MA) têm revelado níveis muito significativos de diferenciação genética, cuja origem tem sido fundamentalmente atribuída a eventos vicariantes decorridos nos períodos Terciário e Quaternário. Apesar destes estudos, muitos dos processos que estão na base desses padrões carecem de uma investigação mais detalhada. Assim, neste trabalho procurou-se investigar os processos de diversificação espaço-temporais de um grupo de anuros endémicos da MA, pertencentes ao grupo de espécies *Phyllomedusa burmeisteri*, através da combinação de ferramentas moleculares e análises baseadas em Sistema de Informação Geográfica.

O estudo da filogenia deste grupo, através de métodos que produzem árvores de genes e árvores de espécies, permitiu recuperar a monofilia de cada uma das espécies previamente definidas morfologicamente. Este trabalho sugeriu ainda que estas espécies estão divididas em dois grupos principais: 1) um formado pelas espécies distribuídas mais ao norte (*P. bahiana* e *P. burmeisteri*) e um outro, 2) formado pelas espécies distribuídas mais ao sul (*P. distincta*, *P. tetraploidea* e *P. iheringii*). Além disso, os resultados mostraram que *P. distincta* e *P. tetraploidea* são espécie-irmãs, fornecendo suporte adicional para a provável origem de *P. tetraploidea* a partir de um processo de autopoliploidização de *P. distincta*. De acordo com as estimativas temporais a divergência entre os dois principais grupos (Norte e Sul) ocorreu há aproximadamente 5 milhões de anos, enquanto dentro dos grupos a diversificação terá ocorrido desde o final do Plioceno e durante o Pleistoceno. Os

padrões de diversificação geográfica e temporal dentro do grupo foram congruentes com os descritos para outros organismos co-distribuídos. Dados paleoclimáticos e geológicos independentes sugerem que fenómenos de vicariância associados com as oscilações climáticas e atividade neotectônica parece ter contribuído para os atuais padrões de divergência dentro do grupo *P. burmeisteri*.

Através de uma abordagem filogenética e filogeográfica, usando diferentes classes de marcadores moleculares, procurou-se aprofundar vários aspetos relacionados com a taxonomia e padrões de variabilidade genética de *P. bahiana* e *P. burmeisteri*. Os resultados apoiaram o reconhecimento das duas espécies definidas anteriormente com base em análises morfológicas, enquanto unidades taxonómicas independentes, revelando ainda a ocorrência de duas unidades evolutivas altamente divergentes em *P. burmeisteri*, uma das quais restrita ao estado do Rio de Janeiro. Os dados genéticos mostraram sinais de mistura entre as duas espécies, mas não corroboraram a existência de uma extensa área de intergradação fenotípica previamente inferida. Para investigar os padrões de estrutura populacional em *P. burmeisteri* foram especificamente desenvolvidos 12 microssatélites para esta espécie. A análise dos microssatélites através de métodos Bayesianos de agrupamento multilocus e análises multivariadas revelaram quatro grupos geneticamente distintos. Os padrões revelados pelos microssatélites foram discordantes com os obtidos através da análise do mtDNA, com exceção do grupo inferido para o estado do Rio de Janeiro. A coincidência entre estes dois tipos de marcadores forneceu um suporte adicional para o reconhecimento do papel desta área topograficamente complexa do estado do Rio de Janeiro como um microrefúgio do Pleistoceno. No geral, estes resultados sugerem que os padrões espaciais de estrutura genética em *P. burmeisteri* refletem os efeitos combinados de eventos de vicariância antigos e fluxo gênico atual.

Com o objetivo de investigar em detalhe a filogeografia de *P. distincta*, foi utilizada uma abordagem integrativa acoplando métodos filogeográficos convencionais e os baseados em testes estatísticos de hipóteses com técnicas de modelação paleoclimática. Os nossos resultados indicaram a presença de duas linhagens de *P. distincta* altamente divergentes. De acordo com estimativas demográficas, foi sugerido que a linhagem do Sul sofreu uma expansão populacional recente durante o Holoceno. O teste de modelos demográficos históricos alternativos usando uma análise ABC favoreceu um cenário de vicariância antiga (meados do

Pleistoceno), com uma expansão populacional moderada durante o Holoceno. O modelo projetado para o Último Máximo Glaciar corroborou a hipótese de fragmentação do habitat como a explicação mais provável para a diversificação das duas linhagens reveladas pelos dados moleculares.

Um importante objetivo deste trabalho foi testar a hipótese de que a área de distribuição de espécies poliploides está associada a regiões climaticamente instáveis e a habitats mais extremos. Para isso, foi feita uma comparação entre as características ambientais de nicho da espécie poliploides (*P. tetraploidea*) com as das suas espécies diploides filogeneticamente mais aparentadas (*P. distincta* e *P. iheringii*), utilizando técnicas de modelação de nicho ecológico e análises multivariadas. Ambas as análises mostraram que as espécies diploides ocorrem em áreas de menor amplitude térmica e evapotranspiração quando comparadas com as espécies tetraploides, sugerindo assim que *P. tetraploidea* ocupa um espaço ecológico coincidente com as áreas de habitat mais extremas. Curiosamente, os nossos resultados corroboraram a localização da zona híbrida natural entre *P. distincta* e *P. tetraploidea* reportada anteriormente, enquanto a possibilidade de hibridação entre as espécies diploides parece ser extremamente reduzida.

No seu conjunto, os resultados obtidos realçam a importância do estudo de vários tipos de marcadores moleculares e a combinação de diferentes metodologias e ferramentas de análise para a identificação de padrões espaciais e temporais de variação genética, mostrando que os processos de diversificação biológica numa das áreas mais biodiversas do mundo possuem um elevado nível de complexidade.

Summary

Patterns of biological diversification in rich biodiversity areas such as the Tropics and Neotropics, have been the target of a growing number of studies. Recent phylogeographic studies of organisms endemic to the Atlantic Forest (MA) have revealed very significant levels of genetic differentiation, whose origin has been mainly attributed to vicariant events occurred in the Tertiary and Quaternary periods. Despite these studies many of the processes that generated these patterns require further investigation. Thus, this study sought to investigate the spatio-temporal processes of diversification of a group of frogs' endemic from the MA, belonging to *P. burmeisteri* species group, combining molecular tools and GIS-based analyses.

The phylogenetic study of this group, based on both gene trees and multilocus species trees, allowed to recover the monophyly of each species previously defined morphologically. This work also revealed two highly supported groups including: 1) *P. bahiana* and *P. burmeisteri* (northern species), and 2) *P. distincta*, *P. tetraploidea* and *P. iheringii* (southern species), given in addition support to the sister-taxa relationship between *P. tetraploidea* and *P. distincta*. Estimates of divergence suggested a major split within the *P. burmeisteri* group at approximately 5 Myr, while the main clades were originated between ~ 0.4 and 2.5 Myr, spanning the late Pliocene and Pleistocene. Patterns of geographic and temporal diversification within the group were congruent with those uncovered for other co-distributed organisms. Independent paleoecological and geological data suggest that vicariance associated with climatic oscillations and neotectonic activity may have driven lineage divergence within the *P. burmeisteri* group. The integrative phylogenetic and phylogeographic multilocus approach used to study *P. bahiana* and *P. burmeisteri* supported the

recognition of the two currently defined species, providing evidences for one novel and highly divergent evolutionary unit within the range of *P. burmeisteri*, restricted to the Rio de Janeiro state. Genetic data showed signs of admixture between both species, but do not corroborate the previously inferred wide area of phenotypic intergradation. Additionally, based on the twelve polymorphic microsatellite loci specifically developed for *P. burmeisteri*, were used to investigate population structure patterns of this species. Both Bayesian multilocus clustering assignment methods and multivariate analyses were concordant in revealing four genetically distinct groups of populations. The pattern of population substructure was broadly discordant with that revealed by previous mtDNA analysis, with exception of the group uncovered for the Rio de Janeiro state. This coincidence across markers in revealing an evolutionary unit of *P. burmeisteri* in the region of Rio de Janeiro constitute an additional support for the recognition of the role of this topographically complex area as a Pleistocene microrefugia. Overall, these results suggested that the broad-scale patterns of spatial genetic structure of *P. burmeisteri* are still reflecting the combined effects of ancient vicariance events and current gene flow.

In order to investigate in detail the phylogeography of *P. distincta* we used an integrative approach coupling traditional and statistical methods of phylogeographic analysis with species paleodistribution modelling. This study confirmed the presence of two divergent lineages with coherent geographic distribution within *P. distincta* range. Demographic estimates suggested that the Southern lineage has experienced a recent population expansion that started in the Holocene. The testing of alternative historical demographic models using an ABC analysis favoured a scenario of ancient vicariance (mid-Pleistocene) with a moderate population expansion during the Holocene. The LGM paleodistribution model corroborated that habitat fragmentation may explain the diversification of the two lineages revealed by the molecular data.

One additional important goal of this work was to test the hypothesis that polyploidy distribution range is associated with climatically unstable regions and harsher habitats. For that we compared the niche environmental characteristics of the polyploid (*P. tetraploidea*) with those of its phylogenetically close diploid species (*P. distincta* and *P. iheringii*), using ecological niche model techniques and multivariate analyses at fine-scale level. Both analyses showed that diploid species' occur in areas with both lower thermal amplitude and evapo-transpiration than the tetraploid species, suggesting that *P. tetraploidea* occupies an ecological space

coincident with areas of harsher habitat. Interestingly, the results obtained in this study corroborated previously documented natural hybrid zone between *P. distincta* and *P. tetraploidea*, while an extremely reduced chance of hybridization between diploid species' was found.

Taken together, these results highlight the importance of studying several types of molecular markers, and the combination of different methodologies and tools for the identification of spatial and temporal patterns of genetic variation, and revealing the complexity of the processes of diversification in one of most biodiverse areas of the world.

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ABC	Approximate Bayesian Computation
APE	Análise de Parcimônia de Endemismo
ARG	Argentina
AUC	Area Under the Curve
BA	Estado da Bahia
BAF	Brazilan Atlantic Forest
BPP	Bayesian Phylogenetics and Phylogeography
ENM	Ecological Niche Modelling ou Modelos de Nicho Ecológico
IBGE	Instituto Brasileiro de Geografia e Estatística
DAPC	Discriminant Analysis of Principal Components
DD	Data Deficient
DNA	Deoxyribonucleic Acid
ES	Estado do Espírito Santo
IUCN	International Union for Conservation of Nature
LC	Least Concern
LGM	Last Glacial Maximum
MA	Mata Atlântica
MG	Estado de Minas Gerais
MMA	Ministério do Meio Ambiente (Brasil)
mtDNA	Mitochondrial DNA ou DNA mitocondrial
NGS	Next Generation Sequencing ou Sequenciação de Nova Geração
nuDNA	Nuclear DNA ou DNA nuclear
PAR	Paraguai
PR	Estado do Paraná
RJ	Estado do Rio de Janeiro

ROC	Receiver Operating Characteristic curve
RS	Estado do Rio Grande do Sul
SC	Estado de Santa Catarina
SE	Estado do Sergipe
SIG	Sistema de Informação Geográfica
SL	Sampling locality
SNA	Sympatric Northern Area
SNP	Single Nucleotide Polymorphism
SSA	Sympatry Southern Area
SP	Estado de São Paulo
SPCA	Spatial Principal Component Analysis
UES	Unidades Evolutivas Significativas
UMG	Último Máximo Glacial
URU	Uruguai

Capítulo 1

Introdução Geral

1.1 Biologia evolutiva: origem e organização da diversidade biológica

As abordagens integrativas, em geral, fazem parte da raiz das Ciências Naturais, *sensu lato Naturalis Historiae*. Este tipo de abordagem, que foi prática comum durante muitos séculos, só recentemente voltou a ser reconhecida como valiosa, contrariando a tendência para uma crescente especialização. Isto é particularmente importante hoje em dia devido aos grandes avanços científicos em todas as áreas e à imperiosa necessidade de juntar as partes para a construção de um todo. Neste sentido, procurou-se neste estudo abordar vários aspetos da biologia evolutiva usando a informação de várias disciplinas, como a filogenia, filogeografia e genética populacional em combinação com modelos de nicho ecológico, que por sua vez reúnem a informação de várias outras disciplinas, como a geologia, climatologia, botânica, entre outras.

1.1.1 Filogenia e delimitação de espécies

Desde a criação do termo "filogenia" por Haeckel (1866), a representação das relações evolutivas entre grupos de organismos através de árvores filogenéticas passou por diversas transformações (Dayrat, 2003), podendo atualmente incorporar vários tipos de informação biológica, como características fisiológicas, comportamentais, ambientais e morfológicas, e mais recentemente moleculares. O uso de ferramentas moleculares teve fortes implicações na organização hierárquica da diversidade biológica e dos processos subjacentes, tendo conduzido a uma ampla reflexão conceptual sobre a unidade biológica utilizada para categorizar e estimar biodiversidade. Durante muito tempo, o conceito de espécie não foi definido literalmente como um conceito, mas sim com base em diferentes critérios (de Queiroz 1998). Em termos gerais, o conceito de espécie mais comum, e que prevaleceu durante muitos anos, é o conceito biológico, que a define como grupos de organismos que se reproduzem naturalmente e geram descendentes viáveis (Wright 1940; Dobzhansky 1950). Este conceito pode ainda incluir o isolamento reprodutivo, que se traduz na ausência de cruzamentos interespecíficos com base em propriedades intrínsecas como as barreiras geográficas (Mayr 1942). No entanto, com o avanço da biologia molecular e métodos de análise recentes, este conceito

biológico de espécie tem sido amplamente criticado, sobretudo à medida que os resultados revelam uma maior aproximação com o *continuum* evolutivo descrito por Darwin (1859). Além do conceito biológico de espécie, cerca de outros 20 "conceitos" foram já propostos com base em diferentes evidências biológicas, como por exemplo, na monofilia (conceito filogenético), grupos de indivíduos que habitam nichos diferentes (conceito ecológico), grupos de indivíduos morfologicamente distintos (conceito morfológico) e segmentos populacionais cuja preservação maximiza o seu potencial de sucesso evolutivo (i.e. Unidades Evolutivas Significativas, UES - conceito evolutivo), entre outros (ver referências e revisões em de Queiroz 1998, 2007). Este autor identificou diversas lacunas na aplicação de um conceito *per se* para definir "espécie" (tal como tradicionalmente designada por outros autores). Com efeito, de acordo com de Queiroz (2007) o processo de especiação é gradual. Sumariamente, ele começa por considerar que as populações ao serem separadas por uma barreira ao fluxo gênico, sem ação da seleção natural e deriva genética, conduzirá à diferenciação de duas linhagens-filhas. Com o tempo, estas linhagens-filhas vão adquirir propriedades diferentes, que podem ser consideradas como evidências biológicas que preenchem os critérios adotados pelos diferentes conceitos de espécie acima mencionados. Durante o processo de especiação, a acumulação de diferentes propriedades secundárias não ocorrem necessariamente ao mesmo tempo nem através de uma ordem regular e, por conseguinte, diferentes conceitos de espécie podem entrar em conflito, em especial durante os estágios iniciais da especiação (Fig. 1.1).

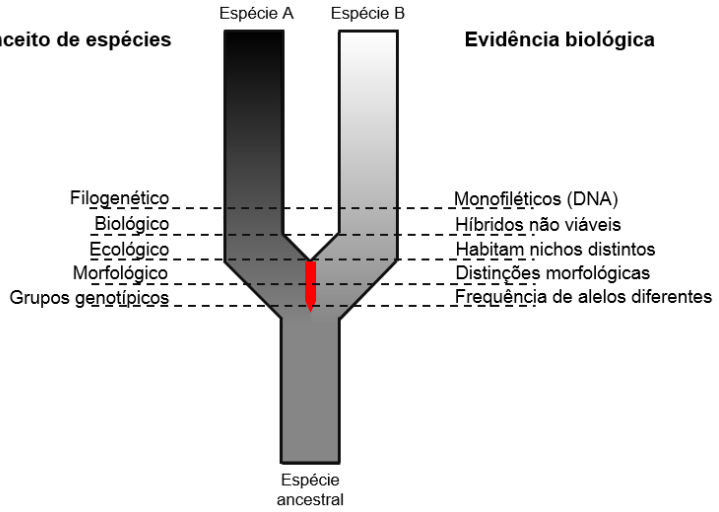

Fig. 1.1 Processo de separação e divergência em um diagrama simplificado, conceitos de espécie e as respetivas evidências biológicas (adaptado de Queiroz 1998; De Queiroz (2007). Barreira para o fluxo génico representada em vermelho.

Assim, antes de se realizar estudos sobre as forças que influenciam a distribuição geográfica da variação genética dos indivíduos, primeiro é necessário que exista um conhecimento prévio sobre a filogenia e os limites específicos dos organismos-alvo. A falta de informação filogenética detalhada é particularmente crítica no caso de espécies estreitamente relacionadas. Durante muito tempo a taxonomia dos grupos e/ou complexos de espécies foi baseada apenas em informações fenotípicas. Apesar de esta prática se manter, nomeadamente em regiões de elevada biodiversidade como as Tropicais e as Neotropicais, tem-se procurado encontrar métodos alternativos baseados em informações contidas no ácido desoxirribonucleico (DNA). No entanto, o fácil acesso às informações sobre as relações evolutivas entre os indivíduos/populações com base no DNA exerceu uma influência direta no aumento da documentação da diversidade biológica, nomeadamente, através de técnicas simplificadas para identificar espécies que, contudo, podem apresentar inconsistências aliados a uma serie de problemas metodológicos (San Mauro & Agorreta 2010). O método mais popular, desde então, se resume na utilização da informação de um gene mitocondrial (mtDNA) como uma forma de identificar espécies, seguindo o mesmo princípio de um código de barras (do inglês, DNA *barcoding*) (Hebert *et al.* 2003). Embora este método constitua uma ferramenta valiosa, a análise do mtDNA isoladamente é muito criticada, devido às suas características intrínsecas, como a herança uniparental, ausência de recombinação e, ainda, a possibilidade de conter mais do que um tipo de genoma numa mesma célula (i.e. heteroplasmia) (ver revisão em Avise 2009). Estas características aliadas ao menor efetivo populacional fazem com que o mtDNA em comparação com o genoma nuclear seja mais afetado pela natureza estocástica do processo genealógico, que leva à perda de muitas das variantes genéticas. Deste modo, além da informação contida no mtDNA torna-se também essencial analisar genes nucleares (nuDNA) para a identificação/delimitação de espécies. No entanto, é importante realçar que muitas vezes as informações contidas em genes mitocondriais e nucleares são discordantes. Para além do processo estocástico de separação de linhagens, a ocorrência de fluxo génico e a retenção de polimorfismo ancestral ao nível dos genes nucleares estão entre as causas mais bem documentadas (Machado & Hey 2003; Pinho *et al.* 2008; Toews & Brelsford 2012; Sousa-Neves *et al.* 2013).

Nos últimos anos, o desenvolvimento de métodos de análises filogenéticas

que acomodam a informação de múltiplos *loci* têm contribuído para minorar os problemas associados à aplicação de métodos usados para a análise de um só *locus* a um "super-gene" (concatenação), que pode incorporar diferentes tipos de informação. Estes métodos passaram por uma grande atualização recentemente, ao incorporar a teoria da coalescência (Kingman 1982), podendo dividir-se em métodos que delimitam "espécies" e métodos que estimam árvores de "espécies". Ambos métodos são complementares em termos gerais, enquanto os primeiros testam a partição da diversidade genética em unidades taxonómicas evolutivas (OTUs) através de testes de probabilidades *a posteriori*, as árvores de espécies procuram estimar as relações filogenéticas e tempos de divergência entre linhagens/taxa diferenciados (Leaché & Rannala 2011; Carstens *et al.* 2013). A maior parte destes métodos exigem uma definição *a priori* das unidades evolutivas (i.e. com base em evidências morfológicas, ecológicas, genéticas, entre outras).

1.1.2 Filogeografia convencional e estatística

O principal objetivo das análises filogeográficas é combinar a informação biogeográfica com árvores filogenéticas para inferir os processos históricos e contemporâneos que moldaram a arquitetura genealógica atual das populações e das espécies estreitamente relacionadas (*lato sensu*, Avise 2009). Embora essa reunião formal da filogenética e da genética de populações com a biogeografia histórica, através do termo "Filogeografia" (Avise *et al.* 1987), seja considerada recente, esta disciplina e o número de trabalhos publicados nessa área cresceram exponencialmente nas duas últimas décadas (ver revisões em Avise 2009; Hickerson *et al.* 2010). A acumulação destes trabalhos foi aos poucos transformando a perspetiva sobre os marcadores moleculares utilizados nos estudos filogeográficos, que inicialmente se baseavam apenas no mtDNA (Avise *et al.* 1987). No entanto, hoje em dia sabe-se que a utilização adicional do nuDNA e o aumento do número de *loci* são essenciais, devido à natureza estocástica do processo genealógico (ver detalhes na secção anterior). Sendo assim, a utilização de múltiplos genes neste tipo de análises é fundamental, por exemplo, para diminuir a incerteza das estimativas de tempos de divergências entre populações (Edwards & Beerli 2000).

Em termos convencionais, os padrões filogeográficos são interpretados de forma descritiva, a partir da reconstrução de árvores e/ou redes de haplótipos,

podendo incorporar estimativas de alguns parâmetros demográficos, tais como o tamanho efetivo da população, taxas de migração, crescimento ou o declínio populacional e, ainda, tempos de divergência (e.g. Watterson 1975; Tajima 1989; Wakeley & Hey 1997). Estas informações em conjunto permitem compreender a história das populações de uma forma geral, no entanto, o entendimento dos padrões evolutivos mais complexos requer a aplicação de métodos de análise mais rigorosos e, ao mesmo tempo, flexíveis. Com efeito, a implementação de inferências estatísticas no campo da filogeografia foi fundamental para a compreensão da dinâmica das populações ao longo do tempo. Através de métodos estatísticos baseados na teoria de coalescência, as genealogias são tratadas como critérios de transição para se obter estimativas de parâmetros demográficos biogeograficamente informativos, ao contrário da utilização das genealogias de genes estimadas para inferir diretamente a história demográfica (ver revisão em Nielsen & Beaumont 2009). A este propósito, Hudson (2002) desenvolveu um método (ms) capaz de simular genealogias envolvendo uma população assumindo diferentes cenários evolutivos, em que é possível variar, por exemplo, a taxa de fluxo génico, taxa de recombinação, tempos de divergência e o tamanho do efetivo populacional. Mais tarde, Hickerson *et al.* (2007) possibilitaram que pares de espécies/populações fossem comparados através de um modelo hierárquico via computação bayesiana aproximada (do inglês, Approximate Bayesian Computation, ABC). O reconhecimento verificado nos últimos anos da importância destes testes estatísticos de hipóteses encontra-se refletido no aperfeiçoamento e complexidade dos modelos incorporados em programas cada vez mais simplificados na ótica do utilizador, resultando numa crescente publicação de artigos usando esta metodologia (Amaral *et al*. 2013; Tsai & Carstens 2013).

1.1.3 Distribuição geográfica das espécies

Embora tanto os processos evolutivos quanto os ecológicos ocorram num contexto geográfico, a história evolutiva dos organismos a longo prazo está mais associada com a dimensão geográfica do que com a ecológica propriamente dita (Peterson *et al.* 1999). Deste modo, uma vez que fatores climáticos e físicos atuaram em algum momento no processo de diversificação e na dinâmica das populações, a capacidade de armazenar, mapear e analisar dados espaciais através dos Sistemas

de Informação Geográfica (SIG), tornou-se rapidamente um atrativo fundamental para o campo da biologia evolutiva e da conservação da diversidade biológica (ver revisões em Swenson 2008; Alvarado-Serrano & Knowles 2014). Esta integração ocorreu, principalmente, através do uso de modelos de previsão de distribuição das espécies (ou Modelos de Nicho Ecológico).

A possibilidade de se prever a distribuição geográfica de uma determinada espécie no presente, no passado e até mesmo no futuro, vem sendo apresentada como uma importante fonte de validação externa para os estudos filogeográficos. Este tipo de modelo de previsão é construído a partir da correlação entre a distribuição das espécies e diferentes características ambientais (i.e. clima, topografia e cobertura do solo), que fornecem informações sobre o espaço geográfico que engloba as preferências abióticas e tolerâncias ambientais das espécies (i.e. nicho realizado; Soberón & Peterson 2005). As estimativas da dimensão e distribuição geográfica espécies estão cada vez mais sendo utilizadas em diversas áreas, como na sistemática, ecologia, conservação e na filogeografia. As aplicações destes modelos, quando combinado com estudos filogeográficos, permitem contrastar se a localização das populações que contem maior diversidade genética corroboram as potencias áreas de estabilidade climática em períodos glaciares (e.g. Waltari *et al.* 2007), avaliar os principais requisitos ambientais que são responsáveis pela posição e manutenção de zonas híbridas (Martínez-Freiría *et al.* 2008; Tarroso *et al.* 2014) e, ainda, testar a possibilidade de existir diferenças de nicho entre espécies divergentes (e.g. Mccormack *et al.* 2010; ver revisão em Alvarado-Serrano & Knowles 2014).

1.2 Mata Atlântica: "*Terra Brasilis*"

1.2.1 Bioma

A Mata Atlântica (MA) é um dos biomas mais biodiversos e ameaçados da região Neotropical (Myers *et al.* 2000). A sua origem remonta a ~ 100 milhões de anos atrás quando o então supercontinente Gondwana separou-se em grandes blocos de terra e formou, entre outros, o subcontinente denominado América do Sul. Este fenómeno, por conseguinte, desencadeou uma série de eventos geológicos e climáticos ao longo de toda a costa Atlântica que possibilitaram a formação de

grandes cadeias montanhosas, nomeadamente a Serra do Mar, e a abertura de grandes cursos de água, propiciando uma série de habitats e micro-habitats. Esta grande região de floresta tropical e subtropical ocupou no passado cerca de 1,5 milhões de km^2 de toda costa Atlântica da América do Sul, atingindo algumas pequenas regiões a oeste, conhecidas como Mata Atlântica Interior (Fig. 1.2) (Sanmartín & Ronquist 2004; Ribeiro *et al.* 2011).

Fig. 1.2 Mapa parcial da América do Sul representando a cobertura original do bioma Mata Atlântica e os principais rios da bacia do Atlântico Leste. No sentido Norte/Sul: Rio São Francisco, Rio das Contas, Rio Pardo, Rio Jequitinhonha, Rio Doce e Rio Paraíba do Sul, respetivamente. Escala: 1:50.000.000. Fonte: SOS Mata Atlântica.

Uma das suas características mais marcantes é a extrema heterogeneidade ambiental. O bioma é formado por um mosaico vegetacional, onde a fitofisionomia (tipo de formação vegetal) predominante é a Floresta Ombrófila Densa (MA *strictu sensu*). Ao mesmo tempo e em consequência do aumento do deficit hídrico com o afastamento da costa, surgem as formações de Florestas Ombrófilas Abertas, Estacionais Semidecidual e Decidual e, Enclaves de Estepe, assim como na transição para a região subtropical, ao sul, a Floresta Ombrófila Mista (Fig. 1.3). Tais variações estruturais estão intimamente associadas à grande amplitude latitudinal (3º S – 30º S) e longitudinal (35º O – 60º O) de sua distribuição. A MA possui acentuados gradientes ambientais altitudinais (0 – 2,900m de altitude), campos de altitude, e formações de influência marítima, como as restingas e manguezais (IBGE

2012). Recentemente, foi proposto uma subdivisão do bioma em 55 regiões biogeográficas, com base em 19 variáveis ambientais e relevo (ver Ribeiro *et al.* 2011), o que é uma boa ilustração da complexidade existente dentro do que é genericamente chamado de Mata Atlântica.

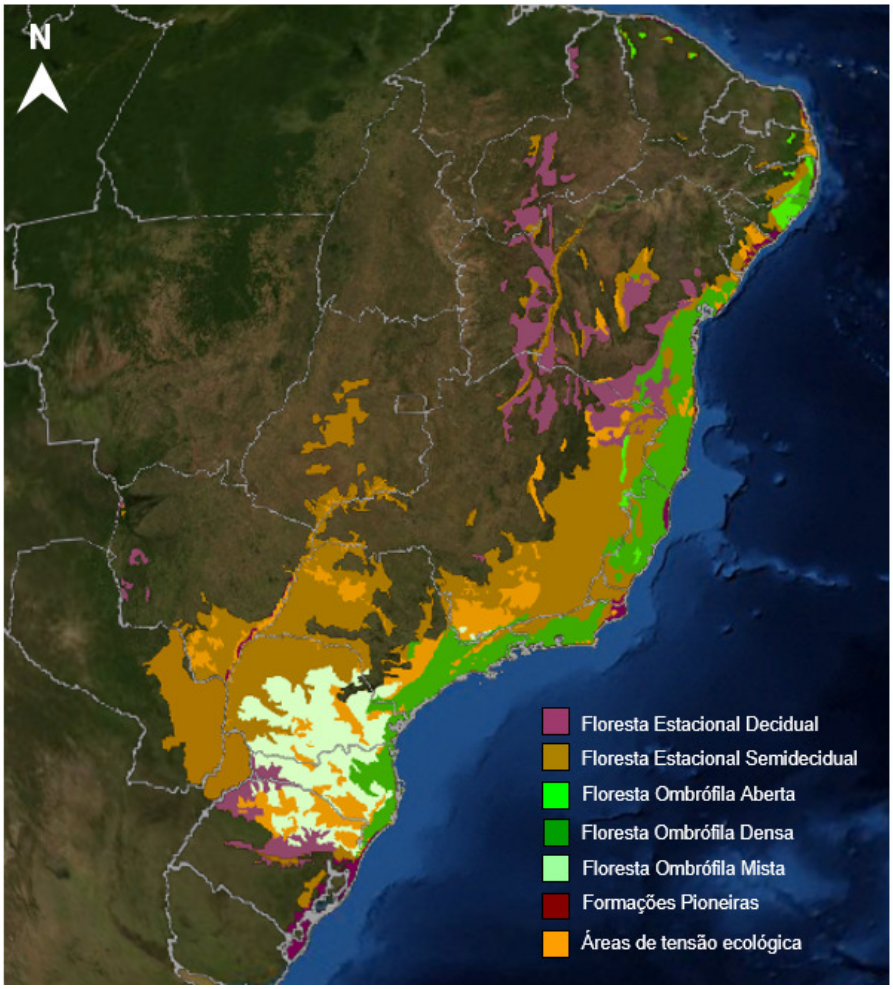

Fig. 1.3 Mapa parcial da América do Sul com a distribuição das diferentes fitofisionomias da Mata Atlântica. Escala: 1:35.000.000. Fontes: IBGE/MMA, 2008.

1.2.2 Biodiversidade e conservação

A Mata Atlântica reúne uma grande diversidade biológica e elevadas taxas de espécies endémicas. Dentre os 34 *hotspots* mundiais de biodiversidade, a MA ocupa o terceiro lugar no ranking em número de espécies de anfíbios (516), quarto em espécies vegetais (20.000) e sexto em aves (936) e mamíferos (312). As taxas de endemismo também são bastante expressivas, rodando os 40% para as espécies vegetais e 63% para os anfíbios (Mittermeier *et al.* 2011). No entanto, estas estimativas ainda estão muito aquém da real diversidade biológica da MA

(Lewinsohn & Prado 2005; Pimm *et al.* 2014). Estes índices têm vindo a aumentar nos últimos anos, principalmente em função do uso de informações ao nível molecular. Com efeito, é importante ressaltar que diversos estudos tem revelado uma quantidade significativa de divergências genéticas profundas em formas/espécies crípticas que até então eram consideradas uma só espécie (e.g. Mata *et al.* 2009; Fusinatto *et al.* 2013; Gehara *et al.* 2013).

Porém, a degradação do bioma vem ocorrendo de forma muito mais veloz do que a produção de conhecimento sobre o mesmo. O uso dos recursos e as alterações antrópicas datam desde a ocupação humana pré-colombiana. Já o início da supressão florestal em larga escala remonta ao século XVI, com a colonização europeia. Neste período iniciaram-se ocupações não integradas à natureza e à exploração dos recursos naturais como o pau-brasil (nome dado à descoberta então "Terra Brasilis"), passando pelo ouro no século XVIII e, ainda, pelo início dos cultivos de cana-de-açúcar, na região nordeste, a partir do XVII e do café, na região sudeste, a partir do XIX. Estes períodos foram seguidos pela industrialização, que se concentrou essencialmente na costa Atlântica, levando a que nos dias de hoje a maior parte da população e das atividades decorrentes da presença humana (Dean 1996; Pádua 2010) estejam concentradas nesta região.

Hoje em dia, 17 estados brasileiros e parte da região leste do Paraguai e da Argentina estão localizados na área de cobertura original da Mata Atlântica (Fig. 1.2). Desta cobertura original, restam cerca de 12,6%, se considerarmos os três países onde ela ocorre (Chebez & Hilgert 2003; Huang *et al.* 2009; Ribeiro *et al.* 2009). Com isto, quase todos os remanescentes já se encontram bastante degradados, sendo que 80% do que ainda restou está distribuído em fragmentos florestais menores do que 50 ha, com variados graus de isolamento (Ribeiro *et al.* 2009). A fragmentação e as atividades como a caça, as queimadas e corte seletivo de árvores têm levado à homogeneização da biota terrestre e ao aumento de estádios sucessionais primários (Groeneveld *et al.* 2009; Lôbo *et al.* 2011; Pütz *et al.* 2011).

1.2.3 Hipóteses e padrões de diversificação biológica

A compreensão dos processos subjacentes à diversidade biológica é o objetivo central da Biologia Evolutiva. Não obstante, os fatores e processos que geraram

tamanha biodiversidade na região Neotropical tem intrigado os investigadores das mais diversas áreas. Por muito tempo, acreditou-se que longos períodos de estabilidade climática/geológica e o isolamento pela distância por si seriam suficientes para justificar a rica biodiversidade Neotropical. No entanto, a partir da década de 60 dois investigadores brasileiros, um geógrafo (Aziz Ab'Saber) e um biólogo (Paulo Vanzolini), começaram a associar as diferentes feições geográficas a indícios de que a MA poderia ter passado por períodos de oscilações climáticas acentuadas, cuja consequência ao nível da modificação do habitat teria originado elevados níveis de biodiversidade. Aziz Ab'Saber, em 1956, levantou a hipótese de que as regiões florestadas teriam sido sujeitas a ciclos de contração/expansão durante o Pleistoceno (Ab'Saber 1977). Esta hipótese, similar à Teoria dos Refúgios Florestais elaborada para explicar os padrões de diversificação e expansão biológica no continente Africano (Moreau 1933), Europeu (Reinig 1935) e da Oceânia (Gentilli 1949), viria também a ser adotada como a principal explicação para os padrões de diversificação biológica da América do Sul, especialmente da Amazónia e Mata Atlântica (Haffer 1969, Vanzolini 1970, Vanzolini & Williams 1981). Mais tarde, surgiram outras hipóteses que sugeriram que o complexo sistema de rios e/ou movimentos geomorfológicos constituíam os principais geradores de diversidade, uma vez que estes funcionariam como barreiras para o fluxo génico provocando o isolamento de populações (Carcraft 1985, Patton *et al.* 1994).

Nos últimos anos, a acumulação de estudos filogenéticos e filogeográficos (ver detalhes na Secção 1.2) sobre os padrões e processos de diversificação biológica na MA, tem revelado a existência de um padrão complexo que envolve a atuação tanto dos refúgios florestais como dos rios e eventos paelogeomorfológicos do Plioceno/Pleistoceno como factores determinantes. Entre os vários estudos realizados, destacam-se o trabalho de Pellegrino *et al.* (2005), que salientou o papel dos grandes rios da costa Atlântica brasileira na diferenciação do complexo *Gymnodactylus darwinii*. De igual modo importante, foi o estudo realizado por Grazziotin *et al.* (2006) que sugeriu que processos orogénicos em combinação com os refúgios florestais teriam atuado no processo de diversificação das serpentes do complexo *Bothrops jararaca* em diferentes escalas temporais. Utilizando aves como organismo modelo, Cabanne *et al.* (2007) encontram evidências de que a evolução do complexo *Xiphorhynchus fuscus* seria resultante da influência dos refúgios florestais durante o Pleistoceno e por isolamento por distância geográfica. É

importante referir que apesar de sugerirem hipóteses diferentes para a diversificação destes organismos, os trabalhos citados encontraram padrões de diversificação similares ao nível geográfico.

No entanto, os primeiros estudos que tentaram explicar o padrão para a distribuição dos organismos ao longo da MA foram feitos através da Análise de Parcimônia de Endemismo (APE). Lynch (1979) sugeriu a divisão deste bioma em três centros de endemismo latitudinais: i) Pernambuco e Bahia; ii) Espírito Santo e São Paulo; iii) Santa Catarina; e dois longitudinais: i) Litoral e ii) Interior tendo como base a ocorrência de 168 espécies endêmicas de anfíbios da MA (Fig. 3). Costa *et al*. (2000) encontrou um padrão bastante similar através da distribuição de mamíferos endêmicos. Anos mais tarde, Carnaval & Moritz (2008) sugeriram que algumas regiões da MA teriam sido mais afetadas pelo Último Máximo Glacial (UGM) e que as áreas mais a norte do Bioma (Norte de São Paulo) poderiam ter sido áreas de floresta mais estáveis (Fig. 1.4). Estes últimos autores utilizaram uma ferramenta baseada em Sistema de Informação Geográfica (SIG) (ver detalhes na seção 1.2) e realizaram uma previsão paleoclimática para a MA ao longo dos últimos 21 mil anos. O modelo previsto foi contrastado com dados palinológicos e genéticos. Em geral, os resultados encontrados por Carnaval & Moritz (2008) são semelhantes aos de Lynch (1979). Ambos sugerem que os padrões de biodiversidade da MA estão divididos em três áreas: i) Norte, Refúgio do Pernambuco; ii) Centro, Refúgio da Bahia; iii) Sul, Refúgio de São Paulo.

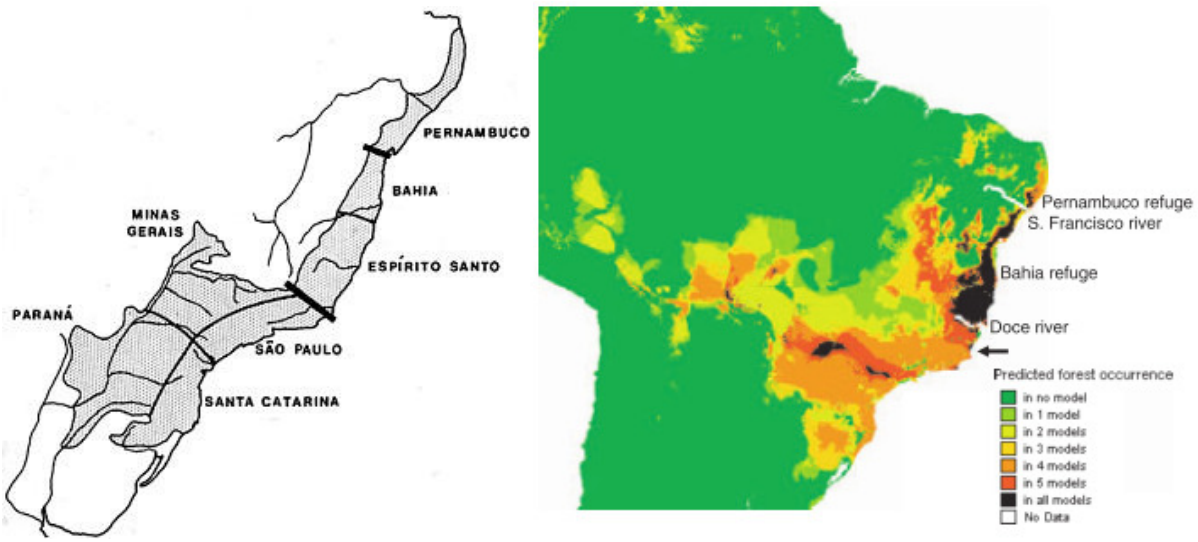

Fig. 1.4 Mapas indicando à esquerda as áreas endêmicas de espécies de anfíbios da Mata Atlântica brasileira de acordo com Lynch (1979); e à direita áreas historicamente estáveis (áreas escuras) de acordo com Carnaval & Moritz (2008).

Mais tarde, outros estudos sugeriram que a região sul da Mata Atlântica foi climaticamente instável em relação à região norte e central, que teriam funcionado como zonas de refúgio para as espécies no final do Pleistoceno (Carnaval *et al.* 2009; Amaral *et al.* 2013). No entanto, a MA tem sido interpretada principalmente como um todo, sendo que a maior parte dos estudos filogeográficos ainda são feitos com base apenas na informação do mtDNA e amostragens espaciais inadequadas quando comparadas aos níveis de dispersão dos organismos. Além disso, extensas revisões revelaram uma maior complexidade da história biológica dessa região e destacaram a necessidade de estudos filogeográficos a uma escala mais fina (Martins 2011; Silva *et al.* 2012). Deste modo, desafios metodológicos como: i) ampliar a gama de marcadores moleculares, nomeadamente, marcadores com taxas de evolução mais rápida e, ainda, ii) a aplicação de métodos de análise multilocus (delimitação de espécies, árvores de espécies e de teste de hipótese de cenários de diversificação) são essenciais para clarificar os padrões biogeográficos dos organismos da MA, que poderão ser cruciais para a delimitação de áreas prioritárias para a conservação.

1.3 Anfíbios

Os anfíbios, em geral, são organismos com altos níveis de estrutura populacional, vulnerabilidade ecológica e limitada capacidade de dispersão, características que os tornam modelos de excelência para investigar os processos de diversificação e, em particular para a identificação de refúgios potenciais e rotas de migração, em resposta a mudanças ambientais do passado e da paisagem (Zeisset & Beebee 2008). Contudo, muitas espécies de anfíbios estão atualmente ameaçadas (~40%) devido a fenómenos relacionados com as alterações das condições ambientais, nomeadamente a desflorestação, a fragmentação e ou a destruição dos habitats (*habitat-split*) e, ainda, pela doença infeciosa (quitridiomicose) causada pelo fungo *Batrachochytrium dendrobatidis*, entre outras ameaças (Becker *et al.* 2004; Cheng *et al.* 2011; Duarte *et al.* 2012). Deste modo, esclarecer os padrões de diversificação, assim como, descrever a biodiversidade ainda não documentada, e explorar a existência de áreas que concentrem variabilidade ao nível genético e as condições adequadas para a sua ocorrência, são essenciais para a manutenção e a conservação das populações de anfíbios.

1.3.1 Género Phyllomedusa

Phyllomedusinae é uma subfamília de sapos-folha Neotropicais da família Hylidae. Esta subfamília de sapos arborícolas contem atualmente sete géneros sendo *Phyllomedusa* o mais biodiverso e com a distribuição mais ampla (ver Faivovich *et al.* 2010). Os sapos-folha caracterizam-se por possuir uma locomoção lenta, pupila vertical, vida arbórea, desova não aquática e por apresentar diversos padrões de cores aposemáticas nas regiões dos flancos e das coxas (Funkhouser 1957). A coloração, neste caso, está associada à presença de uma enorme variedade de peptídeos bioativos únicos na pele destes organismos, que são atualmente reconhecidos como muito importantes para a indústria farmacêutica (Faivovich *et al.* 2010; Calderon *et al.* 2011). De acordo com a última revisão sistemática da subfamília Phyllomedusinae, o género *Phyllomedusa* divide-se em quatro complexos de espécies: *P. tarsius*, *P. burmeisteri*, *P. perinesos* e *P. hypochondrialis*, e em sete espécies distribuídos ao longo da América Central e do Sul (Faivovich *et al.* 2010).

As espécies do género *Phyllomedusa* são conhecidas como “sapos à prova de água”. Sob o ponto de vista fisiológico, estes anuros desenvolveram a capacidade de excretar ácido úrico (uricotélicos) como mecanismo de retenção de água, ao contrário dos demais anfíbios que excretam ureia (Shoemaker *et al.* 1972). Adicionalmente, a presença de glândulas lipídicas associadas a um comportamento sistemático de propagação da secreção lipídica sobre o corpo, utilizando os membros anteriores e posteriores (*wiping behavior*) (Fig. 1.5), seguido de torpor, permitem que estes anfíbios se mantenham impermeabilizados durante o dia. Assim, esta combinação de características fisiológicas e comportamentais resulta em taxas extremamente baixas de perda de água por evaporação quando comparado com outros anuros, e até mesmo em relação a lagartos adaptados ao deserto (Shoemaker & McClanahan 1975).

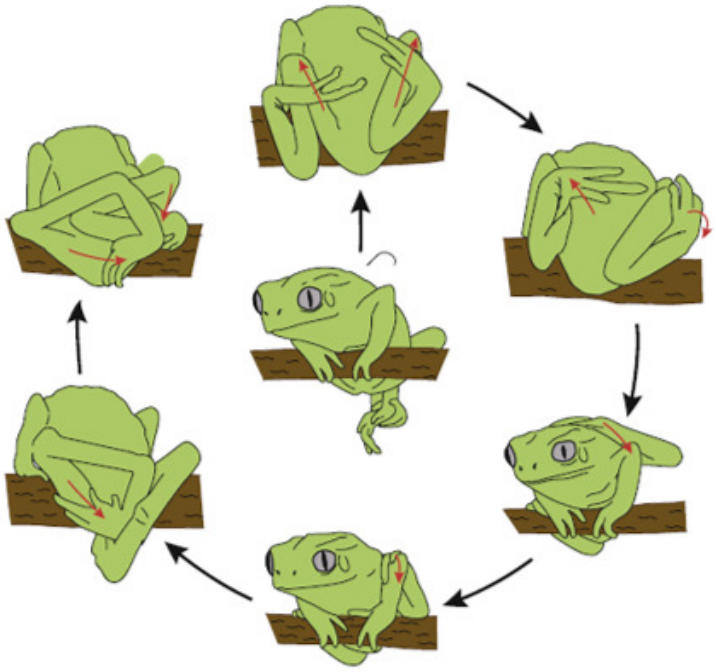

Fig. 1.5 Movimentos usados para espalhar as secreções lipídicas (*wiping behavior*) em *Phyllomedusa sauvagii*. A secreção lipídica é espalhada pela pele por uma série de movimentos sistemáticos, aqui, utilizando os membros posteriores. As setas vermelhas indicam a direção do movimento dos pés. Adaptado de Blaylock *et al.* (1976) e Vitt & Caldwell (2013).

Apesar de ainda existir uma carência de estudos sobre a biologia das espécies de *Phyllomedusa* em geral, Faivovich *et al.* (2010) sugerem que as baixas taxas de perda de água por evaporação estão presentes em todas as espécies de *Phyllomedusa* com base em uma extensa revisão sistemática dos phyllomedusineos. De fato, o *wiping behavior* já foi observado em diversas espécies de *Phyllomedusa*, entre elas, em *Phyllomedusa distincta* (Castanho 1994), *Phyllomedusa bahiana* (J. Zina, dados não publicados) e *Phyllomedusa burmeisteri* (D. Baêta, dados não publicados). Além disso, as espécies de *Phyllomedusa* também apresentam uma resistência protetora significativa em seus estágios iniciais de desenvolvimento, caracterizado pela presença de um modo reprodutivo com base em ovos arbóreos, exotróficos, pendente sobre a água e protegido por folhas (Modo 24 em Haddad & Prado 2005) (Fig. 1.6). Neste caso, apenas os girinos já desenvolvidos atingem os corpos de água, evitando a possibilidade de uma predação massiva, tal como acontece em posturas aquáticas e terrestres.

As espécies de *Phyllomedusa* se defendem dos predadores através de colorações aposemáticas, definidas por cores uniformes e brilhantes ou misturadas com pontos brilhantes ou faixas irregulares, localizadas na região dos flancos e das coxas. Estas áreas coloridas são exibidas intencionalmente na presença de potenciais predadores quando os indivíduos se movem sobre ramos de árvores. No

entanto, se mesmo assim, os indivíduos forem atacados pelos predadores, podem se fingir de mortos (tanatose), ou se engolidos, as secreções tóxicas presentes na pele destes organismos podem provocar a regurgitação (Toledo *et al.* 2011; Sazima 1974).

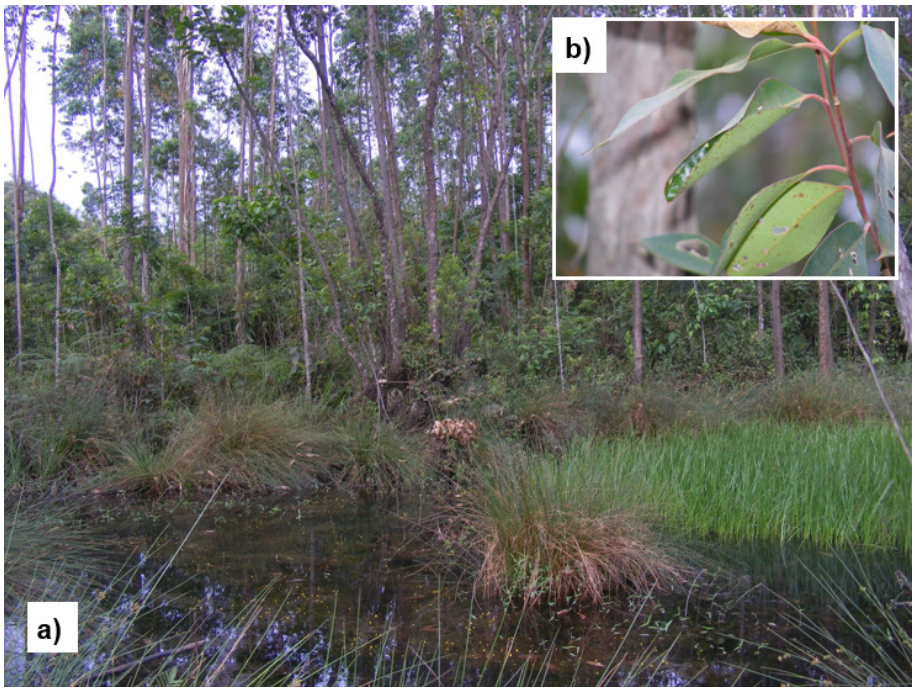

Fig. 1.6 Modo reprodutivo de *Phyllomedusa distincta*: a) poça temporária próximo da floresta; b) desova pendente sobre a água e protegida por folha. Foto: Tuliana O. Brunes (Município de Treviso, Santa Catarina, Brasil, 12/2010).

1.3.2 Espécies de Phyllomedusa do grupo burmeisteri

Dentro do contexto dos anfíbios, o grupo de espécies *Phyllomedusa burmeisteri* constitui um excelente modelo de estudo para a compreensão de aspetos históricos da distribuição das espécies devido à sua ampla distribuição ao longo da Mata Atlântica. Estas espécies estão presentes em tipos variados de habitats, como submontano a montano, Floresta Ombrófila Mista e Densa, Floresta Estacional Semidecidual e Decidual e, ainda, habitats xéricos compostos por savana estépica (ver detalhes abaixo). As espécies de *Phyllomedusa* do grupo *burmeisteri* se distribuem de forma parapátrica e/ou alopátrica, substituindo-se umas às outras ao longo do norte até ao sul da Mata Atlântica e biomas adjacentes. Embora este tipo de distribuição sugira que estes organismos divergiram de forma gradual e/ou vicariante, este grupo inclui uma espécie que se originou a partir de um evento de polipoidização, considerado um mecanismo de especiação instantâneo (ver detalhes abaixo).

Histórico taxonómico

O grupo de espécies de *P. burmeisteri* possui um histórico taxonómico relativamente instável, devido à grande variação fenotípica e ausência de caracteres diagnosticáveis alternativos (ver revisão em Pombal & Haddad 1992). Atualmente, este grupo inclui quatro espécies diploides, *P. burmeisteri* Boulenger, 1882; *P. bahiana* A. Lutz, 1925; *P. iheringii* Boulenger, 1885; *P. distincta A. Lutz*, 1950*;* e uma espécie tetraploide, *P. tetraploidea* Pombal & Haddad, 1992 (Pombal & Haddad 1992; Silva-Filho & Juncá 2006). Dentro do contexto da descoberta da biodiversidade Neotropical, as espécies de *Phyllomedusa* do grupo *burmeisteri* foram primeiramente descritas com base na morfologia, por diferentes investigadores e em períodos distintos da história. A noção de que as espécies supracitadas poderiam constituir um grupo de espécies próximas surgiu a partir da primeira proposta de arranjo filogenético da subfamília Phyllomedusinae (Funkhouser 1957), baseada principalmente em graus de especialização dos pés. Nessa época, a revisão feita considerou apenas *P. burmeisteri*, *P. bahiana* e *P. iheringii* como espécies, sendo que *P. tetraploidea* ainda não tinha sido descrita e *P. distincta* seria considerada uma variação de *P. iheringii*. No entanto, B. Lutz (1950), na mesma altura, considerou todas a formas relacionadas a *P. burmeisteri* como taxas subespecíficos (*P. burmeisteri burmeisteri*, *P. b. bahiana*, *P. b. distincta* e *P. b. iheringii*). Quatro décadas mais tarde, Pombal & Haddad (1992) descreveram *P. tetraploidea* (espécie 4n) tendo, ainda, procedido à organização taxonómica o grupo *Phyllomedusa burmeisteri* com base em um carater fenotípico. Segundo estes autores, o carácter distintivo mais importante entre estas espécies é o padrão de coloração das partes ocultas das coxas, embora, estes mesmos autores refiram-se à existência de indivíduos com padrões de coloração intermediários entre *P. burmeisteri* e *P. bahiana* em uma extensa área. Essa última observação levou os mesmos a manterem o nível subespecífico de *P. b. burmeisteri* e *P. b. bahiana*, enquanto as demais formas foram re-elevadas ao nível específico. A posição taxonómica destas duas espécies foi alterada por Silva-Filho & Juncá (2006) com base em diferenças na morfologia larval e vocalizações gravadas em apenas uma população.

Ao comparar as vocalizações das cinco espécies, Pombal & Haddad (1992) sugeriram que a organização destas espécies estaria dividida em dois grupos: i) um

formado pelas espécies distribuídas ao norte, então consideradas subespécies naquela altura, *P. b. burmeisteri* e *P. b. bahiana* apresentando pulsos isolados e regularmente espaçados, ii) e outro formado pelas espécies distribuída ao sul, *P. distincta*, *P. tetraploidea* e *P. iheringii*, apresentando pulsos aos pares e irregularmente espaçados. A monofilia do grupo *Phyllomedusa burmeisteri* foi recuperada compreendendo as cinco espécies descritas atualmente através de um extenso estudo sobre a filogenia da subfamília Phyllomedusinae, feita com base em vários genes mitocondriais e nucleares (Faivovich *et al.* 2010). Adicionalmente, análises filogenéticas realizadas com um gene mitocondrial sugeriram que este grupo de espécies ter-se-ão divergido durante o Mioceno, além de terem revelado níveis inesperados de estruturação geográfica intraespecífica (Brunes 2009). No entanto, a baixa amostragem ao nível específico não permitiu que este autor contribuísse de fato para as discussões sobre os processos de diversificação das espécies do grupo *P. burmeisteri*, assim como, sobre a origem do evento de poliploidização, deixando claro a necessidade de estudos posteriores.

Distribuição geográfica e tipos de habitats

As espécies de *Phyllomedusa* do grupo *burmeisteri*; *P. burmeisteri*, *P. distincta e P. tetraploidea* são endêmicas da MA, enquanto a distribuição de *P. bahiana* e *P. iheringii* também abrange regiões da Caatinga e do Pampa, respetivamente. A ocorrência de *P. bahiana*, *P. burmeisteri* e *P. distincta* sucede-se geograficamente, de forma parapátrica, desde o norte até ao sul da faixa da MA do Brasil, respetivamente. *Phyllomedusa iheringii* ocorre no bioma Pampa atingindo parte do Uruguai. A espécie tetraploide, *P. tetraploidea*, é a única com distribuição exclusiva no interior-sul da MA (MAI), ocorrendo no interior dos estados de São Paulo e do Paraná até o leste da Argentina e Paraguai. Embora ocorram principalmente em áreas com distribuições não-sobrepostas, áreas de intergradação fenotípica entre *P. bahiana* e *P. burmeisteri* e, ainda, uma área de hibridação natural entre *P. distincta* e *P. tetraploidea* já foram previamente reconhecidas (Pombal & Haddad 1992; Haddad *et al.* 1994) (Fig. 1.7). Estas espécies ocupam regiões de bordadura de florestas, próximo a corpos de água e, provavelmente, se adaptam bem à perturbação antrópica moderada.

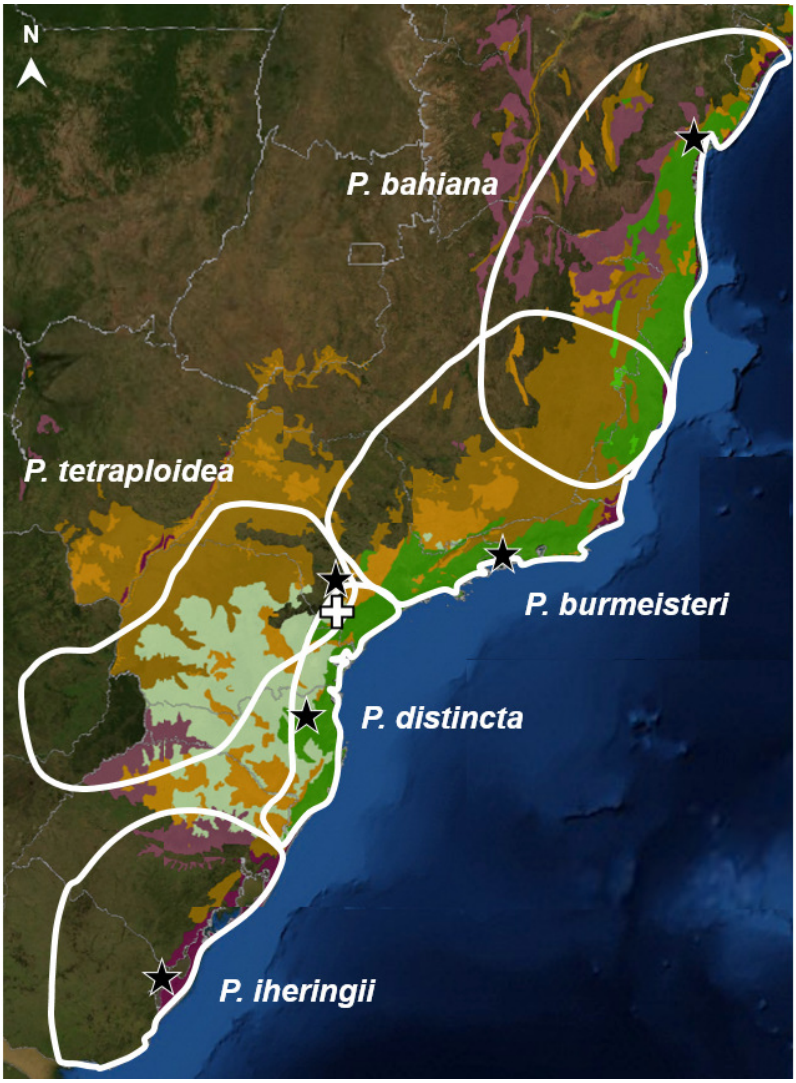

Fig. 1.7 Mapa parcial da América do Sul apresentando a distribuição geográfica das espécies de *Phyllomedusa* do grupo *burmeisteri*. A área de sobreposição entre *P. bahiana* e *P. burmeisteri* representa uma aproximação da zona de intergradação fenotípica. Cruz branca indica o local onde *Phyllomedusa distincta* e *Phyllomedusa tetraploidea* hibridam (Haddad *et al.* 1994). Estrelas pretas indicam as respetivas localidade-tipo. Ao fundo, estão representadas os tipos de fitofisionomias da Mata Atlântica (ver legenda na Fig. 1.2). Ver descrição detalhada de cada espécie abaixo. **Escala: 1:38.000.000.** Fonte: SOS Mata Atlântica.

Phyllomedusa burmeisteri foi descrita em 1882 pelo naturalista britânico George Albert Boulenger com o espécime-tipo coletado na floresta da Tijuca, Município do Rio de Janeiro, RJ, Brasil. Em vida, apresenta um padrão de coloração das partes ocultas das coxas constituído por manchas arredondadas amarelas sobre fundo azul. Ocorre em áreas ocupadas por Florestas Ombrófila Densa e Floresta Estacional Decidual, desde o estado do Espírito Santo (ES) até o sul de São Paulo (SP) (Fig. 1.8). Atualmente possui um estatuto de conservação “pouco preocupante”, LC (do inglês, *Least Concern*, IUCN 2010).

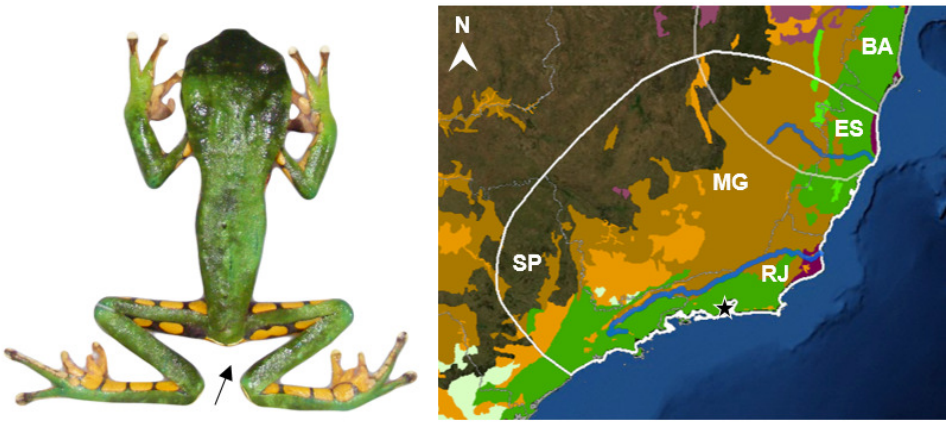

Fig. 1.8 Exemplar de *Phyllomedusa burmeisteri*, respetivo padrão de coloração das partes ocultas da coxa (seta preta) e distribuição ao longo de diferentes estados brasileiros e fitofisionomias da Mata Atlântica (ver legenda na Fig. 1.2). Estrela preta indica a localidade-tipo. Linhas azuis representam os principais rios da bacia do Atlântico Leste incluídos dentro da distribuição da espécie (ver detalhes na Fig. 1.1). Linha com transparência representa parte da distribuição de *P. bahiana* definindo a provável região onde as duas espécies coocorrem. Foto: Tuliana O. Brunes (Município de Santa Maria Madalena, RJ, Brasil, 28/11/2010).

Phyllomedusa bahiana foi descrita em 1925 pelo médico e investigador brasileiro Adolfo Lutz com o espécime-tipo coletado no Município de Salvador, BA, Brasil. Em vida, apresenta um padrão de coloração das partes ocultas das coxas constituído por um colorido uniforme azul, não existindo sinais de manchas. Ocorre na Floresta Ombrófila Densa, Floresta Estacional Decidual e Semidecidual e, ainda, em habitats xéricos ocupadas pelo bioma Caatinga, desde o estado do Sergipe (SE), atingindo a faixa leste de Minas Gerais (MG) até o estado do ES (Fig. 1.9). Atualmente, por falta de informações adequadas para a avaliação do seu estatuto de conservação, se encontra na categoria “dados insuficientes”, DD (do inglês, Data Deficient, IUCN, 2008).

Phyllomedusa distincta foi descrita em 1950 também pelo médico e investigador brasileiro Adolfo Lutz com o espécime-tipo coletado em “Rio Vermelho”, provavelmente, numa localidade próxima ao Município de São Bento do Sul, SC, Brasil. Em vida, apresenta um padrão de coloração das partes ocultas das coxas constituído por um colorido uniforme vermelho, não existindo sinais de manchas. É a menor das espécies do grupo e ocorre em parapatria com *P. tetraploidea* no sudeste de SP. Ocorre principalmente em áreas ocupadas Floresta Ombrófila Densa, desde o sul do estado de SP até o norte do estado do Rio Grande do Sul (RS), sendo, provavelmente a única espécie do grupo com distribuição restrita à MA *stricto sensu* (Fig. 1.10). Atualmente possui um estatuto de conservação “pouco preocupante”, LC (IUCN, 2004).

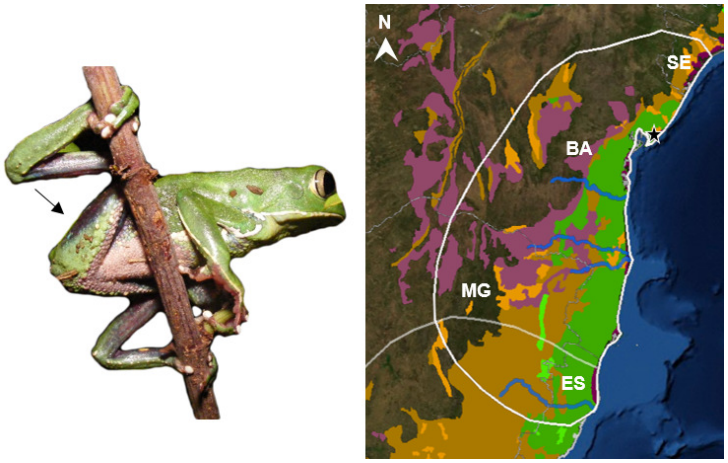

Fig. 1.9 Exemplar de *Phyllomedusa bahiana*, respetivo padrão de coloração das partes ocultas da coxa (seta preta) e distribuição ao longo de diferentes estados brasileiros e fitofisionomias da Mata Atlântica (ver legenda na Fig. 1.2). Estrela preta indica a localidade-tipo. Linhas azuis representam os principais rios da bacia do Atlântico Leste incluídos dentro da distribuição da espécie (ver detalhes na Fig. 1.1). Linha com transparência representa parte da distribuição de *P. burmeisteri* definindo a provável região onde as duas espécies ocorrem em simpatria. Foto: Tuliana O. Brunes (Município de Acajutiba, BA, Brasil, 26/01/2011).

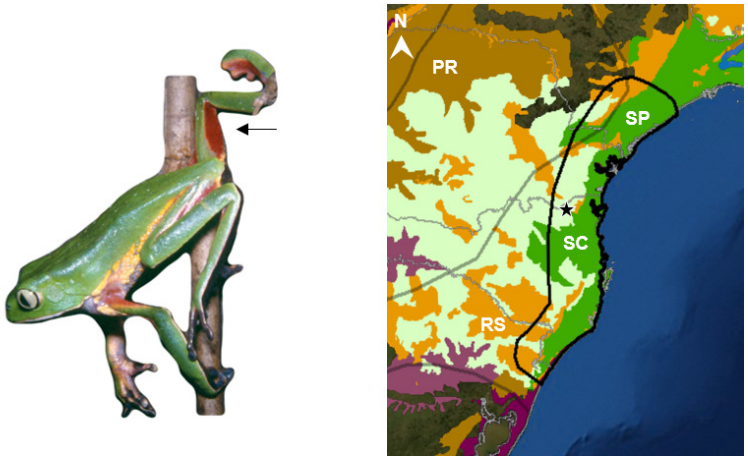

Fig. 1.10 Exemplar de *Phyllomedusa distincta*, respetivo padrão de coloração das partes ocultas da coxa (seta preta) e distribuição ao longo de diferentes estados brasileiros e fitofisionomias da Mata Atlântica (ver legenda na Fig. 1.2). Estrela preta indica a localidade-tipo. Linha com transparência representa parte da distribuição de *P. tetraploidea* definindo a provável região onde as duas espécies ocorrem em simpatria. Foto: Célio F.B. Haddad (Município de Ribeirão Branco, SP, Brasil).

Phyllomedusa tetraploidea foi descrita em 1992 por dois herpetólogos brasileiros José P. Pombal Júnior e Célio F. B. Haddad com o espécime-tipo coletado em *Holambra II*, Município de Paranapanema, SP, Brasil. Em vida, apresenta um padrão de coloração das partes ocultas das coxas constituído por riscos irregulares azuis-escuros em fundo laranja. É a única espécie do grupo que possui o conjunto cromossómico quadruplicado (4n). A origem dessa espécie ainda não foi esclarecida, sendo que hipóteses envolvendo *P. distincta* e *P. iheringii* em um processo de autopoliploidização ou alopoliploidização já foram sugeridas (Pombal & Haddad 1992). Ocorre em parapatria com *P. distincta* no sudeste de SP, onde estas duas espécies hibridizam formando híbridos triploides (ver detalhes abaixo). É encontrada em áreas de Floresta Ombrófila Mista, também conhecida como Floresta

de Araucárias. Também ocorre na Floresta Estacional Semidecidual e Decidual, desde o estado de SP até o leste do Paraguai e da Argentina (Fig. 1.11). Atualmente possui um estatuto de conservação “pouco preocupante”, LC (IUCN, 2004).

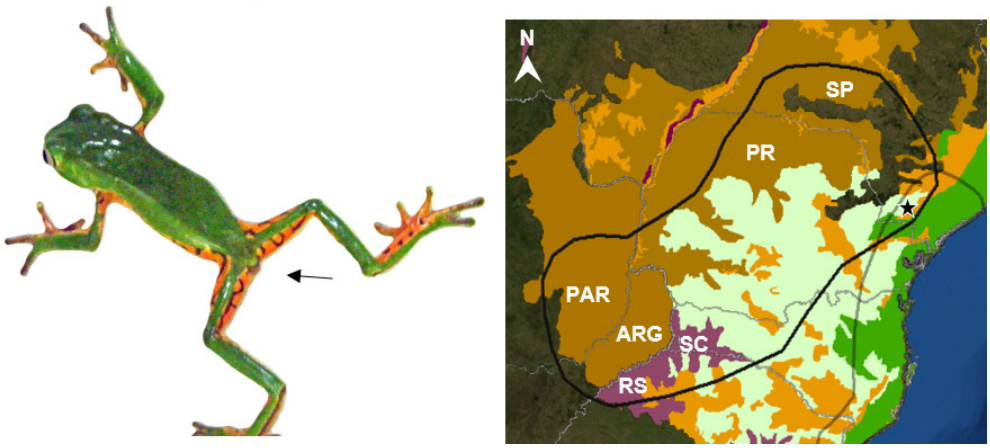

Fig. 1.11 Exemplar de *Phyllomedusa tetraploidea*, respetivo padrão de coloração das partes ocultas da coxa (seta preta) e distribuição ao longo de diferentes estados brasileiros, leste do Paraguai e da Argentina. Ao fundo, as fitofisionomias da Mata Atlântica (ver legenda na Fig. 1.2). Estrela preta indica a localidade-tipo. Linha com transparência representa parte da distribuição de *P. distincta* definindo a provável região onde as duas espécies ocorrem em simpatria. Foto: Tuliana O. Brunes (Município de Tibagi, PR, Brasil, 19/12/2009).

Phyllomedusa iheringii foi descrita em 1885 também pelo naturalista britânico George Albert Boulenger com o espécime-tipo coletado na fronteira sul da lagoa dos Patos, Município de São Lourenço do Sul, RS, Brasil. Em vida, apresenta um padrão de coloração das partes ocultas das coxas constituído por barras azuis escuras sobre fundo laranja. Ocorre em faixas de Floresta Estacional Semidecidual e Decidual, no entanto, a sua distribuição se concentra no bioma Pampa, caracterizado por vegetação reconhecida como savana estéptica, desde a região central do estado do RS até o Uruguai (Fig. 1.12). Atualmente possui um estatuto de conservação “pouco preocupante”, LC (IUCN, 2004).

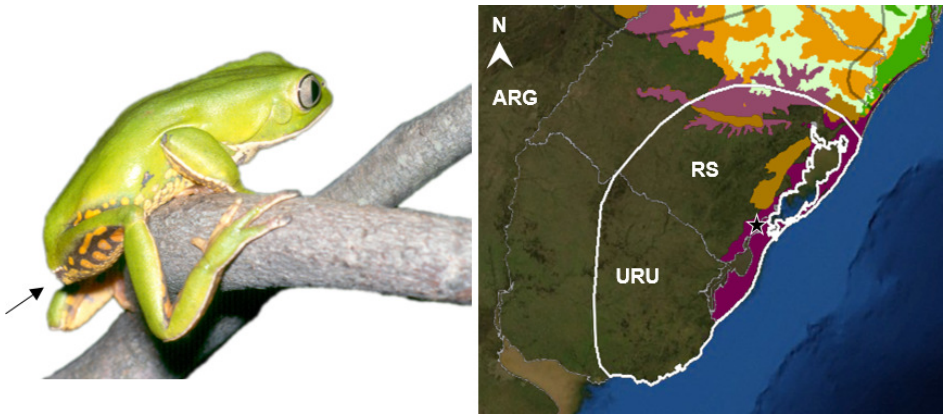

Fig. 1.12 Exemplar de *Phyllomedusa iheringii*, respetivo padrão de coloração das partes ocultas da coxa (seta preta) e distribuição ao longo de diferentes estados brasileiros e leste do Uruguai (URU). Ao fundo, as fitofisionomias da Mata Atlântica (ver legenda na Fig. 1.2). Estrela preta indica a localidade-tipo. Linha com transparência representa parte da distribuição de *P. tetraploide* e *P. distincta*, oeste para leste, respetivamente. Foto: Célio F.B. Haddad (Município de Santa Maria, RS, Brasil).

Área de intergradação fenotípica

Com base no padrão de coloração das partes ocultas das coxas, Pombal & Haddad (1992) observaram um padrão clinal noroeste-sudoeste entre os indivíduos de *P. bahiana* e *P. burmeisteri*, cuja a área de distribuição representa quase metade da área total ocupada pelas restantes espécies do grupo de *P. burmeisteri*. Estes autores observaram uma tendência para a fixação de morfótipos específicos nos extremos da respetivas áreas de distribuição de *P. bahiana* e *P. burmeisteri*, e a presença de indivíduos com padrão de coloração intermediário na região central das mesmas, nomeadamente, em várias localidades dos estados do ES e MG. Com base nestas observações estes autores sugeriram a ocorrência de uma extensa área de intergradação fenotípica (Fig. 1.13).

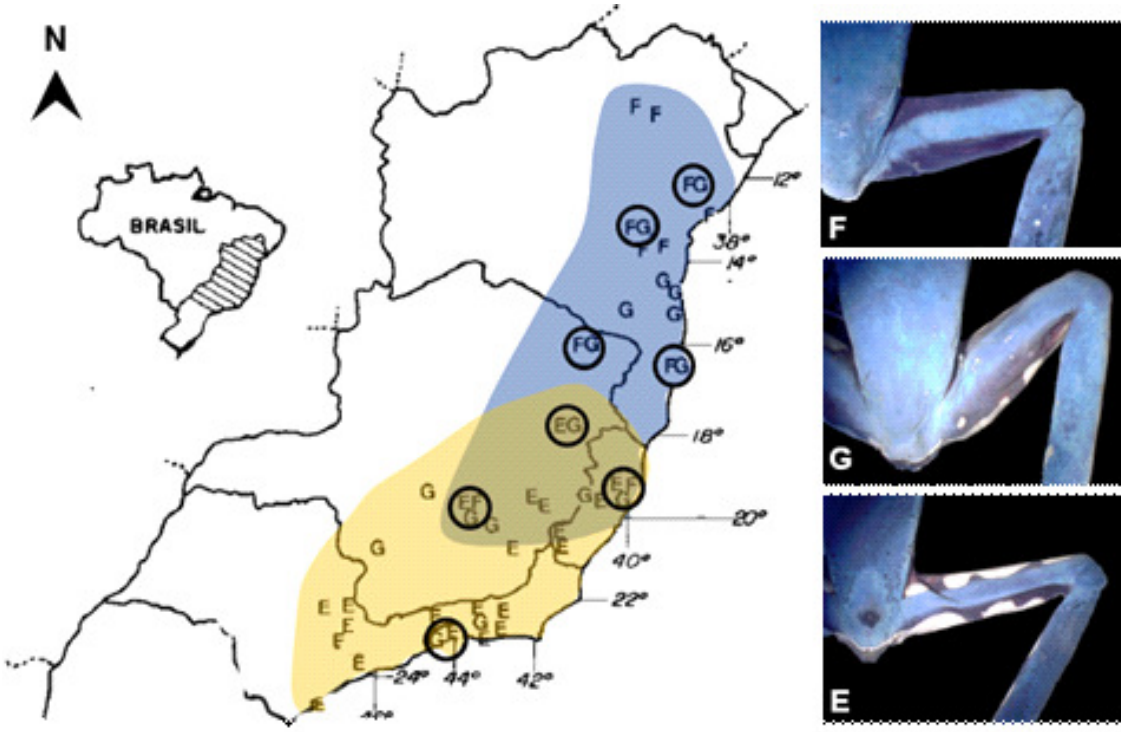

Fig. 1.13 Mapa da distribuição geográfica e do padrão de coloração das partes ocultas da coxa de (F) *Phyllomedusa bahiana*, (G) da forma intermediária entre *P. bahiana* e *P. burmeisteri* e (E) de *P. burmeisteri* (adaptada de Pombal & Haddad, 1992). Círculos representam zonas de simpatria entre os morfótipos. Espécimes depositados na Coleção Herpetológica do Museu Nacional - Universidade Federal do Rio de Janeiro, Município do Rio de Janeiro, RJ, Brasil. Fotos: Délio Baêta.

No entanto, a presença de morfótipos específicos de ambas as espécies dentro desta hipotética extensa área de intergradação, levanta questões pertinentes relativamente à taxonomia destas espécies, uma vez que o padrão de coloração das partes ocultas das coxas é o único caracter utilizado para a classificação destas espécies.

Poliploidização: origem e os potenciais benefícios de uma espécie poliploide

A poliploidização é frequentemente considerada um mecanismo "instantâneo" de especiação devido à ocorrência de isolamento pós-zigótico em consequência da esterilidade do híbrido triploide (ver revisões sobre o tema em Otto & Whitton 2000; Mable 2004; Abbott *et al*. 2013). O seu impacto pode também incluir efeitos sobre mecanismos de isolamento pré-zigótico, como por exemplo, em espécies de anuros em que as formas tetraploides produzem cantos de acasalamento que têm uma frequência de repetição de pulso mais baixa do que as formas diploides das quais se originaram (Gerhardt 2005). Espécies poliploides podem ter origem a partir de eventos de autopoliploidização, mediante a duplicação genómica de uma única espécie ou por aloploidização, envolvendo diferentes genomas através de hibridação (Stebbins 1950). Os eventos de poliploidização têm sido associados a áreas climaticamente instáveis, sujeitas a alterações drásticas ao longo do tempo. Deste modo, segundo expectativas teóricas, espécies poliploides podem apresentar benefícios eco-adaptativos e apresentarem uma maior tolerância a condições ambientais severas quando comparado com as espécies progenitoras diploides (Stebbins 1950). Este fenómeno é considerado mais comum em plantas do que em animais, embora um número relativamente elevado de espécies animais poliploides são já conhecidas, especialmente em grupos de animais ectotérmicos como peixes, répteis e anfíbios (Mable 2004). No caso dos anfíbios, são conhecidos atualmente diversos exemplos de espécies poliploides, como por exemplo nos complexos de espécies de *Rana esculenta*, *Bufo viridis*, *Phyllomedusa burmeisteri*, *Odontophrynus americanus* e *Hyla versicolor* (ver revisões em Mable *et al*. 2011; Evans *et al*. 2012).

A origem da espécie tetraploide dentro do grupo *Phyllomedusa burmeisteri* ainda não foi esclarecida. A este propósito foi sugerido por Pombal & Haddad (1992) que *P. tetraploidea* poderá ter resultado de um processo de autoploploidização recente a partir de *P. distincta*. Para a elaboração desta hipótese, estes autores basearam-se na distribuição geográfica das demais espécies do grupo, os tipos de vocalização e, particularmente, as evidências sobre a ocorrência de hibridização atual entre *P. tetraploidea* e *P. distincta* com a presença de indivíduos triploides estéreis ou com fertilidade baixa (Haddad *et al*. 1994). No entanto, estes autores não descartaram a possibilidade de *P. tetraploidea* se ter originado a partir de um evento de alopoliploidização entre *P. distincta* e *P. iheringii* com base na semelhança da

vocalização (ausência de barreira pré-zigótica) e na possibilidade destas duas espécies possuírem uma zona de contacto na região sul do estado de SC. No entanto, estas três espécies apresentam distribuições divergentes tanto ao nível geográfico quanto do tipo de habitat, com *P. tetraploidea* apresentando uma distribuição exclusivamente no interior sul da Mata Atlântica ocupada por áreas de Floresta Ombrófila Mista e Floresta Estacional Semidecidual e Decidual (Fig.1.10). Embora as espécies tetraploides sejam consideradas, em termos gerais, mais tolerantes as condições ambientais severas do que as espécies diploides progenitoras, as diferenças no nicho climático destas espécies ainda não foram investigadas. Cerca de dez casos de anuros poliploides já foram descritos na região da América do Sul, entre tetraploides e octoploides (ver revisões em Bogart 1980; Mable *et al.* 2011), sugerindo um papel relevante da poliploidia na especiação dos anfíbios desta região

1.4 Objetivos e organização temática

1.4.1 Objetivos

O objetivo principal desta tese é investigar os processos espaço-temporais responsáveis pelos padrões de diversidade biológica na Mata Atlântica, procurando investigar a história biogeográfica das espécies de *Phyllomedusa* do grupo *burmeisteri* e os processos que originaram a sua divergência. Através da combinação de ferramentas moleculares e análises baseadas em Sistema de Informação Geográfica, especificamente procurou-se responder às seguintes questões/hipóteses: i) uma vez que estes anfíbios anuros são amplamente distribuídos, mas ecologicamente restritos, será que os seus padrões filogeográficos mostram a assinatura genética demográfica de recorrentes oscilações climáticas e concomitantes mudanças de habitat que podem ter promovido a divergência de distintas unidades evolutivas?; ii) será que os padrões de variabilidade genética poderão ser explicados através de modelos baseados na delimitação de áreas de estabilidade versus instabilidade de florestas anteriormente descritos para a MA? iii) será que a ocorrência de uma extensa área de intergradação fenotípica é corroborada por análises moleculares? iv) será que a região sul da MA corresponde a uma área de maior instabilidade durante as oscilações climáticas do Pleistoceno,

apresentando uma estrutura genética atual compatível com colonizações recentes ocorridas durante o Holoceno? v) existe diferenciação climática e ambiental entre os nichos das duas espécies diploides filogeneticamente mais aparentadas com a espécie tetraploide? vi) será que as previsões dos modelos de nicho ecológico corroboram a ocorrência da área de hibridação natural já descrita entre a espécies diploide e a tetraploide e preveem outras áreas hibridação potencial entre os taxa analisados? e, vii) Finalmente, considerando que as populações foram provavelmente submetidas a repetidos ciclos de expansão e contração durante períodos de instabilidade climática, será possível detetar assinaturas de fluxo gênico histórico entre as espécies ou unidades evolutivas intraespecíficas?

1.4.2 Organização da tese

Os temas abordados nesta tese são apresentados em seis capítulos, onde os principais objetivos estão organizados sob a forma de artigos científicos. No Capítulo 1, é apresentada uma introdução geral onde se fornece a informação teórica de base sobre os principais domínios da biologia evolutiva abordados nesta tese, como a filogenia, a filogeografia, a genética populacional e as suas diferentes ferramentas de análise. Além disso, é feita uma breve descrição da área de estudo – Mata Atlântica – apresentando-se o estado da arte das principais hipóteses sobre os padrões de diversificação na Mata Atlântica. Por fim, são apresentadas as principais características ecológicas e biogeográficas do grupo de espécies estudado.

No Capítulo 2, são apresentados e discutidos os resultados sobres a relações filogenéticas das espécies de *Phyllomedusa* do grupo *burmeisteri* obtidos com base na análise de um gene mitocondrial e duas genealogias nucleares e, ainda, as primeiras hipóteses sobre os padrões gerais de diversificação quer ao nível espacial quer ao nível temporal. Considerando os limites da amostragem utilizada em função da área de distribuição das espécies do grupo, procurou-se, numa segunda fase, aprofundar os conhecimentos sobre estes aspetos aumentando de uma forma muito significativa a amostragem. Assim, nos Capítulos 3 e 4, realizou-se um aumento muito significativo da amostragem para três espécies do grupo (*P. burmeisteri*, *P. bahiana e P. distincta*), tendo-se recorrido ao uso de um gene mitocondrial e três genealogias nucleares para estudar os seus padrões filogeográficos.

Em um dos artigos do Capítulo 3, a filogeografia de *P. burmeisteri* e *P.*

bahiana, tendo em consideração a extensa área de intergradação fenotípica e o fato de serem espécies-irmã, foram analisadas em conjunto. Assim, neste artigo, para além dos padrões filogeográficos, foram usados métodos de delimitação de espécies e explorada a potencial existência de diversidade ainda não documentada. A possível existência de hibridação entre *P. bahiana* e *P. burmeisteri* anteriormente sugerida pela extensa variação clinal do padrão fenotípico intermediário foi investigada através da análise dos padrões de variação genética e fenotípica. No caso específico de *P. burmeisteri*, foi desenvolvido um conjunto de 12 microssatélites, obtidos a partir de técnicas tradicionais e, principalmente, de sequenciação de nova geração. Estes microssatélites foram usados para o estudo dos padrões de subestruturação genética desta espécie

No Capítulo 4, procurou-se esclarecer os processos evolutivos responsáveis pela elevada subestruturação e variabilidade genética ao nível do mtDNA encontrada em *P. distincta* no Capítulo 2. Para isso, foi utilizada uma abordagem integrativa entre a filogeografia (convencional e estatística) e os modelos paleoclimáticos de distribuição das espécies. Diferentes cenários evolutivos foram contrastados através de um teste de hipótese multilocus com base em ABC. Os modelos de distribuição histórica foram projetados tanto para o presente como para o Último Interglacial (~120 mil anos) e UGM (~21 mil anos).

Ao contrário dos demais, no Capítulo 5 apresenta-se um estudo exclusivamente baseado em modelos de distribuição das espécies e análises multivariadas espaciais. Este último artigo torna-se particularmente interessante por envolver espécies com diferentes níveis de ploidia. Assim, procurou-se comparar o nicho e a influência das condições ambientais na distribuição de espécies filogeneticamente próximas diploides, *P. distincta* e *P. iheringii* (espécies potencialmente envolvidas na origem da forma tetraploide) e da tetraploide, *P. tetraploidea*, com o objetivo de investigar a influência da ploidia na distribuição das espécies.

No Capítulo 6 é apresentada uma discussão geral dos resultados obtidos nesta tese, onde se procura discutir de uma forma integrada os principais resultados obtidos, em particular o seu contributo para o debate das hipóteses sobre os processos de diversificação biológica da Mata Atlântica. Por fim, são apresentadas de um modo sucinto algumas considerações finais e perspetivas futuras de investigação.

1.4.3 Lista dos trabalhos que integram a tese

1. Brunes TO, Sequeira F, Haddad CFB. & Alexandrino J (2010) Gene and species trees of a Neotropical group of treefrogs: Genetic diversification in the Brazilian Atlantic forest and the origin of a polyploid species. *Molecular Phylogenetics and Evolution*, **57**, 1120–1133.

2. Brunes TO, van de Vliet MS, Lopes S, Alexandrino J, Haddad CF.B. & Sequeira F (2013) Characterization of polymorphic microsatellite markers for the Neotropical leaf-frog *Phyllomedusa burmeisteri* and cross-species amplification. *Genetics and Molecular Research*, **12**, 242-247.

3. Brunes TO, Alexandrino J, Baêta D, Zina J, Haddad CFB & Sequeira F. (2014) Species limits, phylogeographic and hybridization patterns in Neotropical leaf frogs (Phyllomedusinae). *Zoologica Scripta*, **43**, 586-604.

4. Brunes TO, Alexandrino J, Haddad CFB & Sequeira F (*in preparation*) Multilocus microsatellite analysis reveals high spatial genetic structure of *Phyllomedusa burmeisteri*, an endemic leaf-frog of the Brazilian Atlantic Forest.

5. Brunes TO, Thomé MTC, Alexandrino J, Haddad CFB. & Sequeira F (*submitted*) Ancient divergence and recent population expansion in a leaf frog endemic to the southern Brazilian Atlantic forest.

6. Brunes TO, Brito JC, Alexandrino J, Haddad CFB & Sequeira F (*in preparation*) Distinct spatial and environmental distributions in diploid-polyploid Neotropical leaf frogs.

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Capítulo 2

Filogenia e diversificação das espécies de *Phyllomedusa* do grupo *burmeisteri*

2.1 Gene and species trees of a Neotropical group of treefrogs: Genetic diversification in the Brazilian Atlantic Forest and the origin of a polyploid species[1]

2.1.1 Abstract

The Neotropical *Phyllomedusa burmeisteri* treefrog group includes four diploid (*P. bahiana, P. burmeisteri, P. distincta and P. iheringii*) and one tetraploid (*P. tetraploidea*) forms. Here we use mitochondrial and nuclear sequence variation from across its range to verify if recognized morphospecies correspond to phylogenetic clades, examine the origin of the polyploid *P. tetraploidea*, and compare range wide patterns of diversification to those of other BAF organisms. We compared single gene trees with one Bayesian multi-gene tree, and one Bayesian species tree inferred under a coalescent framework. Our mtDNA phylogenetic analyses showed that *P. bahiana*, *P. burmeisteri* and *P. iheringii* correspond to monophyletic clades, while *P. distincta* and *P. tetraploidea* were paraphyletic. The nuclear gene trees were concordant in revealing two moderately supported groups including (i) *P. bahiana* and *P. burmeisteri* (northern species) and (ii) *P. distincta*, *P. tetraploidea* and *P. iheringii* (southern species). The multi-gene tree and the species tree retrieved similar topologies, giving high support to the northern and southern clades, and to the sister-taxa relationship between *P. tetraploidea* and *P. distincta*. Estimates of tMRCA suggest a major split within the *P. burmeisteri* group at 5 Myr (between northern and southern groups), while the main clades were originated between 0.4 and 2.5 Myr, spanning the late Pliocene and Pleistocene. Patterns of geographic and temporal diversification within the group were congruent with those uncovered for other co-distributed organisms. Independent paleoecological and geological data suggest that vicariance associated with climatic oscillations and neotectonic activity may have driven lineage divergence within the *P. burmeisteri* group *P. tetraploidea* probably originated from polyploidization of *P. distincta* or from a common ancestor.

1 This section refers to the published article: Brunes TO, Sequeira F, Haddad CFB, Alexandrino J (2010) Gene and species trees of a Neotropical group of treefrogs: Genetic diversification in the Brazilian Atlantic Forest and the origin of a polyploid species. *Molecular Phylogenetics and Evolution*, **57**,1120–1133.

2.1.2 Introduction

The Brazilian Atlantic Forest (BAF) has lost around 90% of its original area of more than one million square kilometres (Morellato & Haddad 2000; Ribeiro *et al.* 2009). BAF is however among the most biodiverse regions of the world, harbouring one of the largest percentages of endemic species (Myers *et al.* 2000). The evolutionary processes that led to this extraordinarily diverse biome have long intrigued evolutionary biologists, but there are only a few studies unveiling the evolutionary processes that determined biological diversification in this megadiversity hotspot. Recently, some molecular studies have confirmed theoretical predictions that species' biogeographical histories have been impacted by the recurrent isolation and persistence of organisms in forest refugia throughout Quaternary climatic fluctuations – Pleistocene refugia hypothesis (*sensu* Haffer 1969) – (e. g., Cabanne *et al.* 2007; Carnaval *et al.* 2009). Other studies, however, suggested that not only Quaternary climatic fluctuations but also climatic and geologic factors such as major rivers, mountain chains and Tertiary tectonic events associated with the formation of geographical landmarks determined current patterns of genetic diversity and diversification of many BAF taxa (Lara & Patton 2000; Pellegrino *et al.* 2005; Grazziotin *et al.* 2006; but see review in Rull 2008). Forest refugia and geographic barriers could have also acted in combination to produce taxon-specific idiosyncratic patterns of diversification (Grazziotin *et al.* 2006; Cabanne *et al.* 2008). To understand their relative importance in producing biological diversification will require detailed multi-species phylogeographical studies across organisms with distinct life histories. This will be especially relevant given that complex topography shaping the diverse phytophisiognomies of the BAF (Oliveira-Filho & Fontes 2000).

Recent studies analyzing both molecular data and palaeoclimatic models of the BAF (Carnaval & Moritz 2008; Carnaval *et al.* 2009) suggested spatial variation in forest persistence throughout the Pleistocene, predicting a large area of historical forest stability in the central corridor (Bahia) and in other small areas (Pernambuco) along the Brazilian coast, roughly concordant with current centres of endemism and mtDNA diversity patterns of few taxa (Costa *et al.* 2000; Costa 2003; Cabanne *et al.* 2008), including three BAF treefrog species (Carnaval *et al.* 2009). Because the species of the *Phyllomedusa burmeisteri* group are widespread mostly in the BAF, inhabiting submontane to montane areas of rainforest, mixed ombrophilus, semi-

deciduous and deciduous forest, they are appropriate model organisms for understanding diversification mechanisms and biogeographical patterns across the BAF. Five species are currently recognized within the *P. burmeisteri* species group based on external morphology, vocalization, and cytogenetics (Pombal & Haddad 1992; Silva-filho & Juncá 2006): the diploid *P. burmeisteri*, *P. bahiana*, *P. distincta*, *P. iheringii*, and the tetraploid species *P. tetraploidea*. Their parapatric or allopatric species ranges replace each other along the BAF northeastern to southern Brazil, with the southernmost *P. iheringii* reaching the Uruguayan Pampas (Fig. 2.1). The tetraploid species is the only with an exclusively inland distribution in southern BAF, including areas in Argentina, Brazil and Paraguay. These treefrogs occur mainly in forested habitats of the BAF, breeding in ponds near the forest edge. The southern *P. iheringii* is an exception as it may occur and breed in the more open areas of the Pampas biome. Although the morphospecies occur mostly in non-overlapping distributions, areas of phenotypic intergradation between *P. bahiana* and *P. burmeisteri*, and one area of natural hybridization between *P. distincta* and *P. tetraploidea* were previously recognized (Pombal & Haddad 1992; Haddad *et al.* 1994).

Phylogenetic relationships across the subfamily Phyllomedusinae based on several mitochondrial and slowly-evolving nuclear genes (exons) supported the monophyly of described *P. burmeisteri* morphospecies, but showing only moderate support to the sister-taxa relationship of *P. tetraploidea* with *P. distincta* (Faivovich *et al.* 2010). Because those results relied on a parsimony analysis of a concatenated dataset, including few representatives per species, were here undertake a rangewide more inclusive multilocus statistical phylogenetic analyses using both mitochondrial and more variable nuclear gene introns under a coalescent framework, to examine species diversification within the *P. burmeisteri* group, particularly the origin of *P. tetraploidea.*

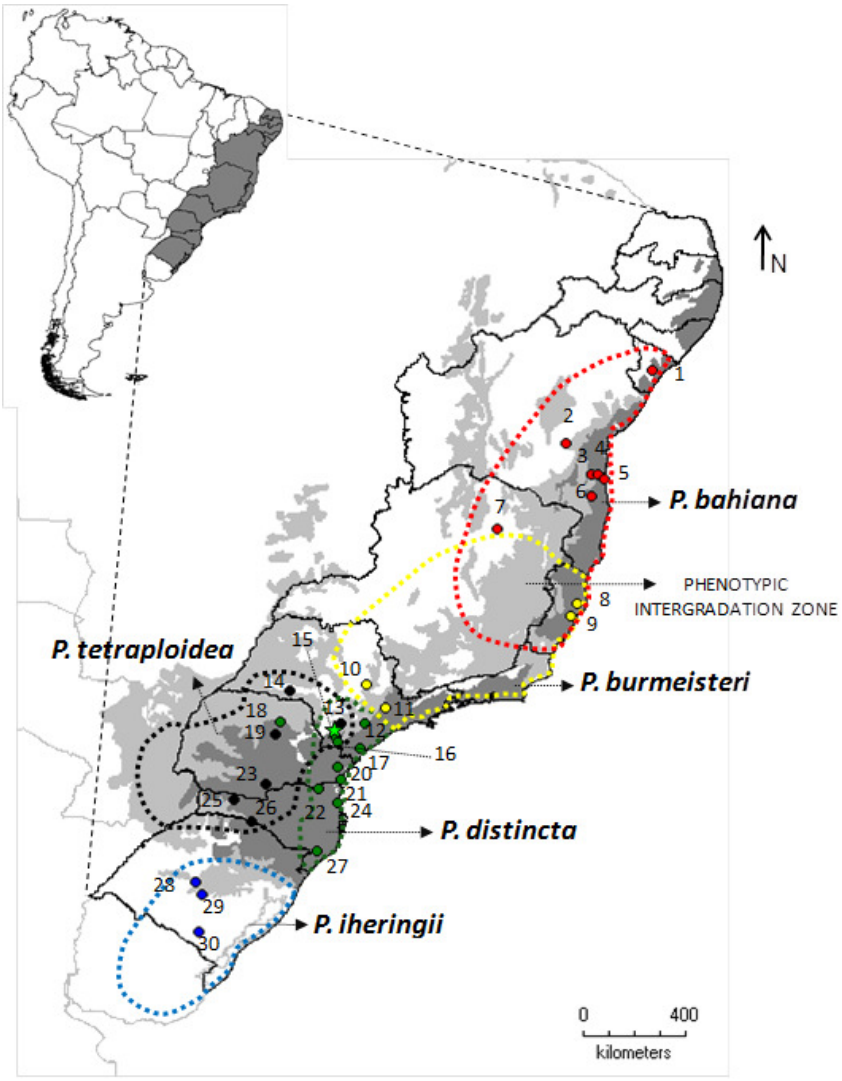

Fig. 2.1 Geographic distribution of *Phyllomedusa burmeisteri* species group with sampling localities numbered 1–30. Brazilian Atlantic Forest original cover: ombrophylous (dark gray) and semi-deciduous (light gray) forests are represented. The green star marks the reported hybridization between *Phyllomedusa distincta* and *Phyllomedusa tetraploidea*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Speciation through polyploidization, once considered relatively rare in animals, has been reported for several vertebrates including anuran amphibians (see reviews in Otto & Whitton 2000; Mable 2004; Gregory & Mable 2005). In most cases, vertebrate polyploid species were originated through autopolyploidization (e. g., Keller & Gerhardt 2001), but with some rare examples of species that resulted from hybridization between distinct species followed by genome duplication, such as in *Xenopus* and *Bufo viridis* subgroup (Tymowska 1991; Evans *et al.* 2004; Stöck *et al.* 2006, 2010). Haddad *et al.* (1994) suggested that *P. tetraploidea* resulted from recent autopolyploidy of *P. distincta*, taking into account their hybrid zone, the geographic distribution of the other species in the group, cytogenetic data and similar breeding vocalizations. This is noteworthy given that differences in ploidy are frequently

accompanied by both distinct geographical distributions and advertisement calls (Stöck 1998; Martino & Sinsch 2002).

Phylogenetic analyses are one of the most widely methods used to trace the evolutionary history of polyploid species. In most cases, mitochondrial DNA was the marker of choice (see several examples in Ptacek *et al.* 1994; Evans *et al.* 2004), given its higher mutation rate and informativeness to examine diversification among closely related species (Pamilo & Nei 1988; Hudson & Coyne 2002). Recently, the additional use of nuclear loci in- creased (e.g., Evans *et al.* 2005; Stöck *et al.* 2010), cautioned by the fear that high levels of recombination could obscure phylogenetic signal (Osborn *et al.* 2003; Evans *et al.* 2005). Recombination should however not be problematic if it is low or absent, and the reconstruction of a coalescent-based species-tree combining nuclear and mitochondrial genes should overcome the putative source of inference errors of a single gene tree due to stochastic variation in the coalescent process of each gene (Bachtrog *et al.* 2006; Edwards *et al.* 2007).

Here we analyze mitochondrial and nuclear sequence variation from across the range of the *P. burmeisteri* species group to verify (i) the hypothesis that recognized morphospecies correspond to phylogenetic clades, (ii) the origin of the polyploid *P. tetraploidea*, and (iii) if range wide patterns of diversification in the group parallel those recently described for other BAF. To this purpose, we compared single gene trees with one multi-gene tree estimated using partitioned Bayesian analysis of concatenated datasets, and one species tree resulting from Bayesian inference under a coalescent framework.

2.1.3 Materials and methods

Population sampling and DNA extraction

In this study we obtained tissue samples from 72 field collected individuals of the *Phyllomedusa burmeisteri* group and one outgroup taxa (*Phyllomedusa boliviana*). We chose this species as outgroup based on published molecular data (Faivovich *et al.* 2010) (Table A.1). We sampled all the five currently recognized species of the group in 30 georeferenced localities from all over the species range (Fig. 2.1, Table 2.1). We removed a portion of liver or muscle prior to specimen fixation in 10% formalin and storage in 100% ethanol. Vouchers are deposited in the Célio F.B. Haddad amphibian collection at Departamento de Zoologia, Universidade Estadual

Paulista "Júlio de Mesquita Filho" (CFBH), Museu de Zoologia da Universidade de São Paulo (MZUSP), Miguel T. Rodrigues collection at Universidade de São Paulo (MTR), and Museu de Ciência e Tecnologia/ Pontifícia Universidade Católica do Rio Grande do Sul (MCT/PUCRS).

Table 2.1 Locality information and sample sizes for 30 populations sampled across the range of the *Phyllomedusa burmeisteri* species group. State abbreviations: SE, Sergipe; BA, Bahia; MG, Minas Gerais; ES, Espírito Santo; SP, São Paulo; PR, Paraná; SC, Santa Catarina; RS, Rio Grande do Sul.

Code	Locality	State	Taxon	N	Longitude	Latitude
1	Areia Branca	SE	*P. bahiana*	1	-37.3153	-10.7578
2	Maracás	BA	*P. bahiana*	4	-40.4308	-13.4411
3	Aurelino Leal	BA	*P. bahiana*	4	-39.5395	-14.5734
4	Uruçuca	BA	*P. bahiana*	5	-39.2844	-14.5931
5	Ilhéus	BA	*P. bahiana*	1	-39.0636	-14.7822
6	Camacan	BA	*P. bahiana*	1	-39.5087	-15.4164
7	Grão Mogol	MG	*P. bahiana*	3	-42.9024	-16.5909
8	Linhares	ES	*P. burmeisteri*	1	-40.0722	-19.3911
9	Aracruz	ES	*P. burmeisteri*	1	-40.2733	-19.8203
10	Rio Claro	SP	*P. burmeisteri*	1	-47.6755	-22.3472
11	Jundiaí	SP	*P. burmeisteri*	1	-46.9439	-23.2496
12	Pilar do Sul	SP	*P. distincta*	1	-47.7164	-23.8131
13	Buri	SP	*P. tetraploidea*	4	-48.5928	-23.7975
14	Assis	SP	*P. tetraploidea*	1	-50.3933	-22.5992
15	Ribeirão Branco	SP	*P. tetraploidea* (6) *P. distincta* (7)	13	-48.7430	-24.3586
16	Iporanga	SP	*P. distincta*	1	-48.7003	-24.5328
17	Pariquera-Açu	SP	*P. distincta*	1	-47.8811	-24.7150
18	São Jerônimo da Serra	PR	*P. distincta*	1	-50.7411	-23.7275
19	Ortigueira	PR	*P. tetraploidea*	1	-50.9494	-24.2083
20	Antonina	PR	*P. distincta*	1	-48.7119	-25.4286
21	Guaratuba	PR	*P. distincta*	3	-48.6324	-25.8694
22	São Bento do Sul	SC	*P. distincta*	1	-49.3786	-26.2503
23	Cruz Machado	PR	*P. tetraploidea*	4	-51.2583	-26.0784
24	Barra Velha	SC	*P. distincta*	3	-48.7657	-26.7222
25	São Domingos	SC	*P. tetraploidea*	1	-52.4550	-26.6314
26	Piratuba	SC	*P. tetraploidea*	2	-51.7719	-27.4197
27	Treviso	SC	*P. distincta*	5	-49.4575	-28.5156
28	Santa Maria	RS	*P. iheringii*	2	-53.8069	-29.6842
29	São Sepé	RS	*P. iheringii*	3	-53.5653	-30.1606
30	Candiota	RS	*P. iheringii*	1	-53.6725	-31.5581

DNA extraction, amplification, and sequencing

Tissue samples were digested in lysis buffer and Proteinase K. Purified whole genomic DNA was obtained using QIA Quick DNEasy columns (Qiagen, Inc.) according to the manufacturer's protocol. We sequenced the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) and two nuclear genes, b-fibrinogen intron 7 (hereafter referred to as b-fibint7) and a segment of exon 2 and intron 2 of the cellular myelocytomatosis (hereafter referred to as C-myc2). We obtained mtDNA sequences for 72 samples, and nuclear sequences for a representative subsample of those for phylogenetic inferences only (15 individuals for both b-fibnt7 and C-myc2).

Sequencing was performed from enzymatically purified PCR products in the ABI Prism BigDye Terminator Cycle sequencing protocol in an ABI PRISM 3130 XL Genetic Analyser and by the Macrogen Corporation sequencing facility (http://www.macro- gen.com). GenBank Accession Numbers: HQ262424–HQ262491.

ND2. Amplification and sequencing of the ND2 gene was accomplished using primers L4437 (Macey *et al.* 1997) and int_Halbo2 (Prado *et al.* 2012, 5'-GTCTAATTTATCCTAAGTTTC-3'). PCR was performed in 25 µl volume using PuReTaq Ready-To-Go PCR Beads (GE Healthcare 2006) with ~50 ng of genomic DNA and 0.2 µM each primer. Amplification conditions consisted of a pre-denaturing step of 3 min at 92 ºC, followed by 40 cycles of a denaturing step of 30 s at 92 ºC, annealing at 50 ºC for 30 s and extension at 72 ºC for 90 s. The final extension was accomplished at 72 ºC for 5 min.

b-fibint7. At a preliminary stage, amplification of the b-fibint7 gene was accomplished using primers FIB-B17U and FIB-B17L (Prychitko & Moore 1997) following the same protocol and amplification conditions described by Godinho *et al.* (2005), but lowering the annealing temperature to 50 ºC. However, because sequencing success was low with these set of primers, sequencing was accomplished using the primers BFXF and BFXR described in Sequeira *et al.* (2006). Finally, due to the lack of success of both primer combinations for *P. iheringii*, the definitive amplification of this gene was accomplished using primer FIB-B17L and the new internal primer BFIint (5'-ATGCATGCCCAGATGTGCAGTAG-3'), while sequencing was performed with BFXR and BFIint. Amplifications were performed under the same PCR conditions described above for ND2, in 25 µl volumes, containing 2 µl 10x reaction buffer (Promega), 2 mM $MgCl_2$, 0.4 mM each dNTP, 0.2 µM each primer, 1 U of Go taq DNA polymerase (Promega) and ~100 ng of genomic DNA.

C-myc2. Amplification of C-myc2 was accomplished using primers cmyc1U (Crawford 2003a) and cmyc3cat (5'-GTTGYTGCTGATCTGTTTGAG-3'). The amplified fragments were sequenced using primer cmyc1U and the new internal primer cmycF (5'-ATAGGAACCTGTAGGACCAG-3'). Amplifications were performed with similar PCR conditions described above for b-fibint7, with the exceptions of annealing temperature (54 ºC).

Sequence variation and genetic analyses

For nuclear sequences we produced two different alignments. First, polymorphic positions in b-fibint7 and C-myc2 were coded using the IUPAC ambiguity codes (nuclear dataset 1). Second, we used three approaches to resolve the haplotype phase of nuclear DNA sequences (nuclear dataset 2): (i) the method of Flot *et al.* (2006) for sequences that were heterozygous for insertions or deletions; (ii) cloning the PCR product for heterozygous samples of *P. tetraploidea*. The purified PCR product was ligated to a plasmid and inserted in *Escherichia coli* using the pGEM-T Easy Vector Cloning kit. Bacteria were grown in solid LB medium, with Ampicilin, X-gal and IPTG. The amplification of each positive clone insert was performed with the universal primers M13 (-20) e M13 (-26). We sequenced the positive clones with an automatic sequencer (ABI PRISM 3130 XL Genetic Analyser); and (iii) the Bayesian algorithm implemented in the PHASE software v. 2.1 (Stephens *et al.* 2001), using known phases of haplotypes determined by the previous method. This analysis was run multiple times (3) with different seeds for the random-number generator and checked if haplotype estimation was consistent across runs. Each run was conducted for 1.0 x 106 iterations with the default values. In both nuclear genes we detected multiple-base insertions or deletions (indels). For analyses we pruned the data assuming that indels likely resulted from a single evolutionary step. We left only the first base of the indel (in the case of insertion) or reduced them to one single step (deletion). We choose this approach rather than completely removing indels because this would significantly reduce the number of polymorphic sites and disregard some of the information contained in the data sets. After these procedures, pruned sequences were aligned manually using BIOEDIT v. 7.0.5.2 (Hall 1999).

For both mtDNA and nuclear fragments (dataset 2), we calculated summary statistics in DNAsp v. 4.50 (Rozas *et al.* 2003). Haplotype sequence divergence (p-uncorrected distance) was estimated in MEGA v. 4.0.2 (Tamura *et al.* 2007). Although we were not able to resolve the haplotype phase for some nuclear DNA sequences of b-fibint7 (three individuals), we used them for calculating the following summary statistics: π (Nei 1987), θ (Watterson 1975) and number of segregating sites (S). To that purpose, each haplotype of unknown phase was randomly separated in two putative haplotypes on the basis of the ambiguity codes i.e., assigning the two alleles at each polymorphic site to one of two sequences (see Borge *et al.* 2005). In the

case of b-fibint7, haplotype diversity and number of haplotypes were estimated removing all unresolved ambiguity sites. To evaluate the possibility of recombination we computed Hudson & Kaplan (1985) Rm statistic (minimum number of recombination events) using DNAsp. Because this statistic is likely to be highly affected by homoplasy, we also used the software PHIPACK (http://www.mcb.mcgill.ca/~trevor) to test for recombination using the pairwise homoplasy index (Φw statistic) of Bruen *et al.* (2006). We used the two available options to estimate such P-values of PHIPACK: an analytical approach and a permutation test (1000 permutations).

Phylogenetic analysis

Gene trees

Phylogenetic analyses of the mitochondrial and nuclear sequence datasets (both nuclear dataset 1 and 2) were performed under Maximum Likelihood (ML), and Bayesian Inference (BI). For ML and BI analyses we first used a more traditional analysis, applying the best fit model of sequence evolution for each of the three genes here analysed. Following recent studies (e.g., Brandley *et al.* 2005; Wiens *et al.* 2010) we also performed a partitioning scheme based on intron and exon regions (C-myc2) and codon positions in the protein-coding ND2 mitochondrial gene, using an independent model for each partition. For C-myc2, analysis of codon position in the protein-coding region (exon) was not performed due extremely low variation. Bayes Factors (BF) were used to discriminate the most appropriate strategy. The models of molecular evolution were selected in jModelTest v.0.1.1 (Posada, 2008) under the Akaike information criterion (AIC, Akaike 1974), following Posada & Buckley (2004).

ML analyses were conducted using RAxML GUI (Silvestro & Michalak 2010), a graphical front-end for RAxML-VI-HPC (Randomized Axelerated Maximum Likelihood Stamatakis 2006). ML with the thorough bootstrap option was run ten times from starting random seeds to generate 1000 nonparametric bootstrap replicates. All ML analyses used the general time-reversible (GTR) with gamma model of rate heterogeneity. Bayesian analyses were performed in MrBayes version 3.0b4 (Ronquist & Huelsenbeck 2003) using two replicate searches with 20 x 10^6 generations each, sampling every 1000 generations. Four MCMC (Markov chain

Monte Carlo) were run simultaneously in each analysis. For the analysis of ND2 by codon position, the nucleotide frequencies were considered fixed for the first and the second positions, and we used the Dirichlet process model for the third position (see Bofkin & Goldman 2007). We employed three strategies for MCMC convergence diagnostics. First, we assessed the convergence of the chains by plotting the log-likelihood values versus generation number using the program Tracer v.1.5 (Rambaut & Drummond 2007). Second, we used the online AWTY program (Nylander *et al.* 2008) to analyse the trace plot of the log-likelihood and the cumulative split frequencies across all post-burn-in generations within each analysis. Third, we checked the standard deviation of split frequencies as a convergence index (<0.001). After chain convergence analysis, we discarded all samples obtained during the first 4 million generations as burn-in. Post burn-in trees from all replicates were combined, and a 50% majority-rule consensus tree was estimated. The frequency of any particular clade in the consensus tree represents the posterior probability of that clade (Huelsenbeck & Ronquist 2001).

Species trees

We used two different methods for species-tree reconstruction: a more traditional partitioned Bayesian analysis, combining mtDNA ND2 with nuclear gene alignments into a single data matrix, consisting of 15 alleles of the 5 ingroup taxa; and the coalescent-based Bayesian species tree inference method implemented in the software *BEAST (Bayesian Inference of Species Tree from Multilocus Data; Heled & Drummond 2010).

For the combined analysis (concatenated dataset), we used the nuclear dataset1 (unphased) together with a reduced mtDNA dataset (same individuals as analysed for nuclear data). The largest possible number of partitions (C-myc2 exon, C-myc2 intron, b-fibint7, and ND2 codon positions) was used as the partitioning strategy (see Brandley *et al.* 2005) (data not shown). Because gene flow can bias the analysis accuracy, we used only representatives from outside of the described hybrid zone between *P. tetraploidea* and *P. distincta*. ML and BI analyses were conducted as described above for individual gene trees, with the exception of the option “perpartition brl” that was used in this case for ML analysis in the soft- ware RAxML.

The software *BEAST (an extension of BEAST v. 1.5.4; Drummond &

Rambaut 2007) also implements a Bayesian MCMC analysis, and is able to co-estimate species trees and gene trees simultaneously (Heled & Drummond 2010). The input file was properly formatted with the BEAUti utility included in the software package, using the same partition scheme of the concatenated analysis. We unlinked substitution model parameters for each partition and specified a relaxed clock with an uncorrelated lognormal distribution (Drummond *et al.* 2006), and a speciation Yule process as tree prior were used. We also used this program to infer the time to the most recent common ancestor (tMRCA) and their credibility intervals (95%) for nodes of interest. It is important to note that in the absence of accurate calibration points from external and independent data (e.g. dated fossil records, known biogeographic events, or paleoclimatic reconstructions), or due to the heterogeneity rate of evolution between the calibrated and uncalibrated taxa, temporal estimates by means of molecular data could be a potential source of inference error, and, therefore, they should be treated with caution (e.g., Heads 2005). In this study, we used the ND2 mutation rate (0.00957 mutations/site/million years) based on a calibration for the Neotropical frog *Eleutherodactylus* (Anura: Leptodactylidae; Crawford 2003b). This calibration has already been applied in some studies of Neotropical amphibians producing congruent results (e. g., Carnaval & Bates 2007; Thomé *et al.* 2010). It has also been shown that mutation rate estimates of amphibian protein-coding mtDNA vary only slightly depending on the marker and group of species (Macey *et al.* 1997; Crawford 2003b). Despite the limitations mentioned above, we believe divergence time estimates are still valuable to provide a proxy for the temporal window of evolutionary diversification in our species group of interest. We performed two independent runs for 150 million generations, sampling every 15,000 generations, from which 10% were discarded as burn-in. To check for convergence we used the program Tracer v1.4 (Rambaut & Drummond 2007). The results were obtained in the TreeAnnotator v1.4.8 (Drummond & Rambaut 2007; http://beast.bio.edu.ac.uk) and visualized in FigTree1.1.2 (Rambaut 2008).

2.1.4 Results

Genetic variation and phylogenetic analyses

Mitochondrial DNA

A fragment of 993 base pairs of the ND2 gene was obtained from 72 individuals of the *P. burmeisteri* group and from one exemplar of *P. boliviana* (outgroup). The ingroup alignment revealed 35 haplotypes defined by 208 polymorphic sites, of which 192 were parsimony informative (Table 2.2). The alignment required a gap with 3 bp-long present in one individual of *P. iheringii* in the position 931–933, which translates the Serine amino acid. Analysis with a distinct evolutionary model for each codon position was preferred (2lnBF = 245) following Kass & Raftery (1995). The Bayesian ND2 tree showed basically unresolved relationships among species of the *P. burmeisteri* group (Fig. 2.2). Five main well-supported clades correspond to the currently recognized morphospecies of the group: *P. bahiana*, *P. burmeisteri*, *P. distincta*, *P. tetraploidea*, and *P. iheringii*. Three of these species are monophyletic, while *P. distincta* and *P. tetraploidea* form a paraphyletic clade. Five haplotypes of *P. distincta* sampled in the hybrid zone between *P. tetraploidea* and *P. distincta* are grouped together with *P. tetraploidea* haplotypes, whereas one haplotype of *P. tetraploidea* sampled in the same area is placed within *P. distincta* clade. All clades showed highly supported sub-clades but only in the case of *P. bahiana* and *P. distincta* a coherent geographic distribution is apparent (Figs. 2.1 and 2.2). ML method yielded an identical topology (not shown).

Table 2.2 Summary statistics, recombination tests and genetic distances (p-uncorrected) within the main mitochondrial clades.

Fragment	**Clade**	**Length**	**Polymorphism**						**Distance**	**Recombination**	
			N	**S**	**h**	% Hd	% π	% θ	% *p-uncorrected*	Rm	Φw
ND2	All	993	72	208	35	96	6.2	4.3	-	-	-
	P. bahiana		19	28	9	87	0.9	0.8	0.88(0.10-2.12)	-	-
	P. burmeisteri		4	33	4	100	1.8	1.8	1.83(0.61-2.22)	-	-
	P. distincta		20	17	7	75	0.8	0.5	0.78(0.10-1.51)	-	-
	P. tetraploidea		23	17	11	92	0.5	0.5	0.50(0.10-1.21)	-	-
	P. iheringii		6	19	4	80	0.7	0.6	0.69(0.10-1.30)	-	-
β-fibint7	All	600-604	36	39	18	97	1.7	1.5	-	4	0.09 (0.07)
	Northern group		12	20	9	100	1.3	1.3	-		
	Southern group		24	15	9	92	0.8	0.7	-		
C-myc2	All	449-454	36	31	19	96	1.4	1.6	-	1	0.60 (0.32)
	Northern group		12	13	10	98	0.7	0.9	-		
	Southern group		24	12	9	93	0.6	0.7	-		

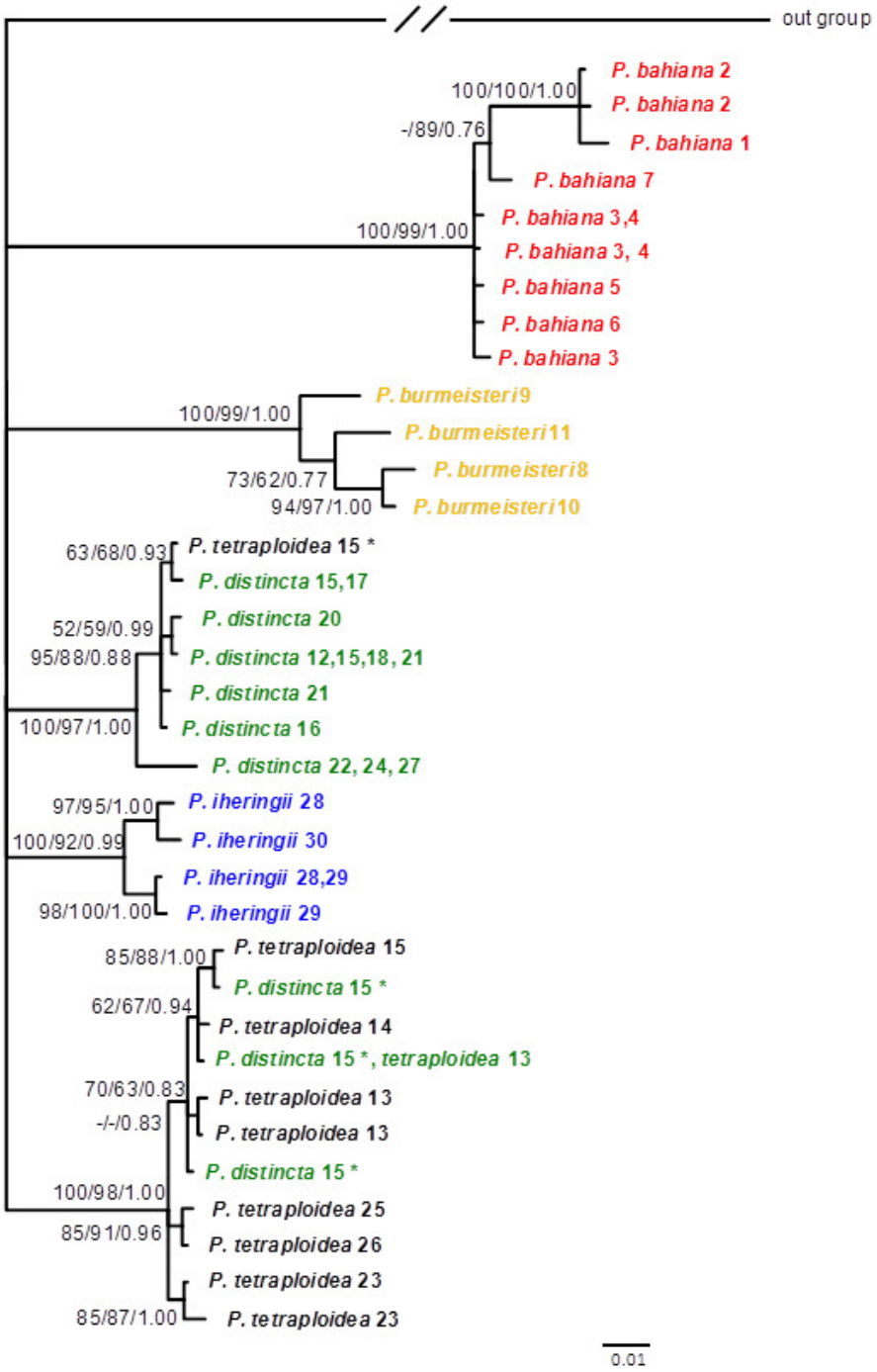

Fig. 2.2 Tree derived from Bayesian analysis of 993 bp of the mitochondrial ND2 sequences in the *P. burmeisteri* species group. Bootstrap values for ML and Bayesian posterior probabilities are given near the branches, respectively. Values under 50% are represented by "–". Haplotype from hybrid zone are represent by "*".

Nuclear DNA

We obtained 36 sequences from 15 samples of the *P. burmeisteri* group. For the nuclear C-myc2, the analysis with a distinct evolutionary model for each partition (exon and intron) was preferred (2lnBF = 7.4) following Kass & Raftery (1995). We found 22 out of 31 polymorphic sites in the ingroup alignment (449–454 bp long) that were parsimony informative. This alignment included four indels 1 bp long. C-myc2

sequences presented one recombinant event according to Hudson & Kaplan (1985) statistics, but the evidence of recombination was not significant according to the PHI test, both using the analytical approach (P = 0.60) or the permutation test (P = 0.32) (Table 2.2). For Bayesian inference, we used the K80 substitution model for the partial exon 2 fragment (134 bp), and TPM2uf substitution model for partial intron 2 (314–320pb) fragment. The Bayesian trees resulting from analysis of datasets 2 and 1 are depicted in Fig. 2.3A and in Fig. A.1, respectively. ML analyses yielded similar topologies for both datasets (not shown).

For the b-fibint7 nuclear fragment, we obtained 36 sequences from 15 samples of the *P. burmeisteri* group. In the ingroup alignment (600–604 bp long) we found 39 polymorphic sites, of which 36 were parsimony informative (Table 2.2). The b-fibint7 fragment alignment also included two indels 1 bp long. b-fibint7 sequences presented four recombinant events according to the Hudson & Kaplan (1985) statistics, but the evidence of recombination was not significant according to the PHI test, both using the analytical approach (P = 0.09) or the permutation test (P = 0.07). The Bayesian trees resulting from analysis of datasets 2 and 1 (Fig. 2.3B and Fig. A.1B, respectively) were similar to ML trees for the same datasets (not shown).

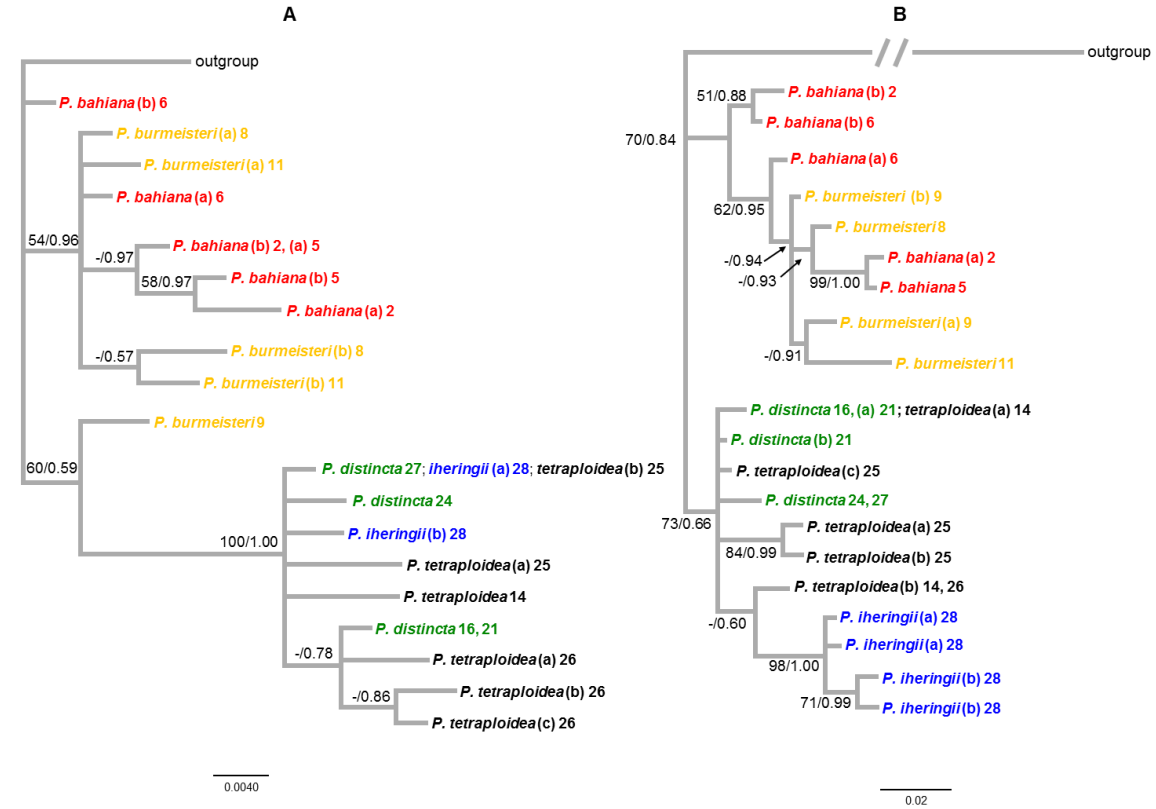

Fig. 2.3 Tree derived from Bayesian analysis of (data set 2) 454 bp of the C-myc2 (A) and 604 bp of the b-fibint7 (B) nuclear sequences in the *Phyllomedusa burmeisteri* species group. Bootstrap values for ML and Bayesian posterior probabilities are given near the branches, respectively. Values under 50% are represented by "–". Letters a, b and c represent different haplotypes found in heterozygote individuals.

Species tree

The two multi-gene species tree methods (combined and *BEAST) recovered a similar topology, unveiling three well-supported monophyletic clades: one corresponding to the more northern distributed species *P. bahiana* and *P. burmeisteri* and the other grouping the southern distributed species (*P. tetraploidea*, *P. distincta* and *P. iheringii*). Within this group the species *P. tetraploidea* and *P. distincta* form a well-supported clade (Fig. 2.4).

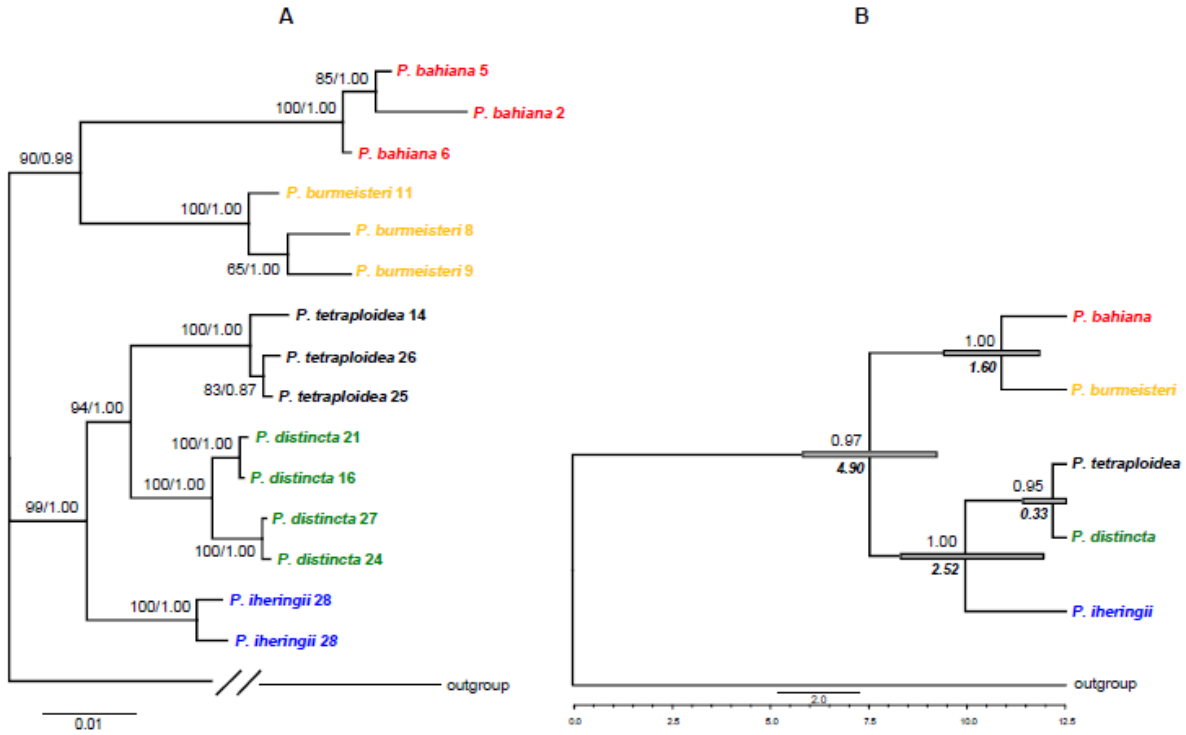

Fig. 2.4 Species tree from the combined mitochondrial and nuclear DNA data: (A) partitioned Bayesian analysis. Bootstrap values for ML and Bayesian posterior probabilities are given near the branches, respectively. (B) Bayesian Inference of Species Trees (*BEAST). Clade posterior probabilities are shown above branches and the mean times to the most recent common ancestor (tMRCA) below branches. The node gray bars represent the 95% confidence intervals (see also Table 2.4).

Genetic distances and divergence time estimates

The mitochondrial average genetic distance (p-uncorrected) obtained within the main clades was 0.88% for *P. bahiana*; 1.83% for *P. burmeisteri*; 0.5% for *P. tetraploidea*; 0.78 for *P. distincta* and 0.69% for *P. iheringii* (Table 2.2). Between the main clades, *p*-uncorrected ranged from 5.3% to 10.2%. The highest divergence was found between *P. bahiana* and all other extant clades (≈10%), while the lowest values were obtained between *P. tetraploidea*, *P. distincta*, and *P. iheringii* (≈5%) (Table 2.3).

Nuclear sequence divergence ranged from 2% to 0.2% in C-myc2, while in b-fibint7 it ranged ≈1.4% to 0.3%. For both nuclear genes the highest divergence was

between the northern species (*P. bahiana* and *P. burmeisteri*) and the southern species (≈2 - 1.5%) while the lowest values were obtained within the southern species group (*P. tetraploidea*, *P. distincta*, and *P. iheringii*; ≈0.2 - 0.6%) (Table 2.3).

Table 2.3 Average sequence divergence (p-uncorrected %) between species of the *Phyllomedusa burmeisteri* group for both mitochondrial and nuclear genes.

Clades	*P. bahiana*	*P. burmeisteri*	*P. distincta*	*P. tetraploidea*	*P. iheringii*
P. bahiana	-	0.39 (0.35)	1.95 (0.84)	2 (0.75)	1.7 (1.1)
P. burmeisteri	10.1	-	1.87 (1.2)	1.93 (1.1)	1.7 (1.45)
P. distincta	10.2	7.5	-	0.33 (0.26)	0.17 (0.61)
P. tetraploidea	9.8	7.8	5.2	-	0.23 (0.35)
P. iheringii	9.4	7.5	5.3	5.4	-

Bayesian coalescent estimates for the time of the most recent common ancestor (tMRCA) were: the main south–north split within *P. burmeisteri* started at around 5 Myr, *P. iheringii* diverged from the other southern species at around 2.5 Myr, *P. burmeisteri* from *P. bahiana* at 1.6 Myr, while the split between *P. distincta* and *P. tetraploidea* occurred around 0.4 Myr (see 95% confidence intervals in Table 2.4).

Table 2.4 Mean time to the most common ancestors (MRCA; 95% confidence intervals in parentheses) corresponding to the nodes of the multilocus coalescent species tree.

*BEAST	MRCA (Myr)
All	5 (3.28 – 6.63)
North group	1.6 (0.65 – 3.05)
South group	2.5 (0.55 – 4.12)
Pdi + Pte	0.4 (0 – 1.06)

2.1.5 Discussion

We used mitochondrial and nuclear sequence variation to infer range wide processes of biological diversification within the *P. burmeisteri* species group. Mitochondrial and nuclear DNA phylogenies were not completely concordant. The first showed that three of the five recognized morphospecies (*P. burmeisteri*, *P. bahiana* and *P. iheringii*) corresponded to monophyletic clades, while *P. tetraploidea* and *P. distincta* were paraphyletic. Nuclear analysis supported a northern group composed of *P. bahiana* and *P. burmeisteri*, and a southern group with unresolved relationships

between *P. distincta*, *P. iheringii* and *P. tetraploidea*. The two inferred species trees were basically concordant, giving high support for northern and southern groups, also supporting *P. distincta* and *P. tetraploidea* as sister-taxa. Our results support the hypothesis that *P. tetraploidea* is an autopolyploid resulting from the lineage of *P. distincta* (Haddad *et al.* 1994); temporal and geographical transitions between species are concordant with phylogeographic breaks uncovered for BAF organisms and with recognized herpetological biogeographic borders within the BAF.

Phylogenetic analysis

Gene trees

Phylogenetic analyses of mtDNA ND2 showed five main clades with high support but with their relationships unresolved. The currently recognized morphospecies *P. bahiana*, *P. burmeisteri*, and *P. iheringii* represent exclusive monophyletic lineages, while *P. tetraploidea* and *P. distincta* are paraphyletic. Haplotypes of five *P. distincta* were placed in a clade that includes all haplotypes of *P. tetraploidea* but one that was placed in the clade with the remaining of *P. distincta* (Fig. 2.2). Paraphyly of closely related species can result from incomplete lineage sorting associated to recent divergence or hybridization (Funk & Omland 2003; Machado & Hey 2003). In the case of *P. tetraploidea* and *P. distincta*, previous work using morphological and cytogenetic data reported a limited zone of hybridization between the two species (Haddad *et al.* 1994). All individuals of *P. distincta* with haplotypes of the *P. tetraploidea* clade and vice versa were sampled in the hybrid sites described by Haddad *et al.* (1994) and are triploid hybrids according to cytogenetic analysis (S. Kasahara and C.F.B. Haddad, unpublished data). Samples of *P. distincta* and *P. tetraploidea* outside of this hybrid zone show exclusive haplotypes from two well-supported diverged clades.

Nuclear C-myc2 and b-fibint7 gene tree topologies were generally similar and contrasted with that of mtDNA (Figs. 2.2 and 2.3). The tree for C-myc2 showed high support for a clade grouping *P. tetraploidea*, *P. distincta*, and *P. iheringii* and a clade including most of the *P. burmeisteri* and *P. bahiana* haplotypes, but the position of four haplotypes of these two species was uncertain (Fig. 2.3a). The b-fibint7 tree showed two exclusive clades with moderate support, one including *P. burmeisteri* and *P. bahiana*, the other including *P. tetraploidea*, *P. distincta*, and *P. iheringii* (Fig. 2.3b).

Nuclear genes therefore showed a subdivision between a northern (*P. bahiana* and *P. burmeisteri*) and southern (*P. tetraploidea*, *P. distincta*, and *P. iheringii*) lineages. The two nuclear genes revealed less power than mtDNA for resolving species-level phylogenetic relationships in the *P. burmeisteri* group. This type of cytonuclear non concordance was described in many other cases of closely related species (Broughton & Harrison 2003; Machado & Hey 2003; Dolman & Moritz 2006; Gonçalves *et al.* 2007; Kotaki *et al.* 2008; Pinho *et al.* 2008) and is not unexpected given that the higher effective population sizes and lower mutation rates of nuclear loci (Slade *et al.* 1994; Moriyama & Powell 1997; Johnson & Clayton 2000) result in coalescent processes acting slower to produce complete lineage sorting (Edwards & Beerli 2000). While monophyly of morphospecies is clearly supported by mtDNA (when 3n hybrids between *P. distincta* and *P. tetraploidea* are excluded), the retention of ancestral polymorphisms could explain the lack of monophyly at nuclear gene trees for the five species of the *P. burmeisteri* group.

Gene flow and introgression between closely related lineages/ species may also cause incongruence between cytoplasmic and nuclear gene genealogies (e.g., Maddison 1997; Nichols 2001; Seehausen 2004; Mallet 2008). A hybrid zone between *P. tetraploidea* and *P. distincta* was documented previously using morphological and cytogenetic data (Haddad *et al.* 1994) and was now confirmed by an mtDNA dataset (present study). It is however difficult to distinguish ancestral polymorphism from gene flow in causing allele sharing in closely related species (revised in Arbogast *et al.* 2002; see also Charlesworth *et al.* 2005; Bull *et al.* 2006; Pinho *et al.* 2008). Concerning nuclear allele sharing between *P. tetraploidea*, *P. distincta*, and *P. iheringii*, the hypothesis of retained polymorphism is favoured by (i) the fact that hybrids between *P. tetraploidea* are *P. distincta* may be sterile or barely fertile (ii) *P. iheringii* is allopatric relative to the other two species (Haddad *et al.* 1994). The morphospecies *P. bahiana* and *P. burmeisteri* are separated by a hybrid zone with morphologically intermediate individuals (Pombal & Haddad 1992). Introgression was not apparent in the mtDNA genealogy which could favour the hypothesis of nuclear gene ancestral polymorphism to explain incomplete lineage sorting. The broadscale nature of the present study (i.e., small sampling of populations and loci) precludes a more detailed exam of alternative hypotheses to explain the dissimilarities between nuclear and cytoplasmic markers.

Species-trees inference combining mitochondrial and nuclear markers

Gene trees may hide the phylogenetic signal of distinct evolutionary units because of retained ancestral polymorphism between species (Maddison 1997; Belfiore *et al.* 2008), which may apply to the nuclear gene data in treefrogs of the *P. burmeisteri* group. Assuming that gene flow did not largely confound true species relationships (see Section 2), we reconstructed species trees under a Bayesian coalescent framework and also using the more traditional method of data concatenation. Although concatenation has been used for inference of multilocus datasets (e.g., Brandley *et al.* 2005; Bossu & Near 2009), recent studies have shown that it may be inconsistent (e.g., Liu & Edwards 2009) and less accurate in recovering species tree topology when compared to Bayesian coalescent-based species tree methods (Heled & Drummond 2010). In the present study, phylogenies resulting from both approaches produced basically similar topologies and posterior probability estimates for species relationships. When compared with gene trees, the species trees give higher support for the northern (*P. bahiana* and *P. burmeisteri*) and southern (*P. tetraploidea*, *P. distincta*, and *P. iheringii*) groups as exclusive evolutionary units. Species trees additionally provided high support for the sister-taxa relationship between *P. tetraploidea* and *P. distincta*, which had low support in the mtDNA gene tree and was not at all apparent in any of the nuclear gene trees topologies.

Overall, phylogenetic relationships for the *P. burmeisteri* species group now recovered are roughly similar to those found by Faivovich *et al.* (2010). Specifically, the results from our much expanded dataset support the morphospecies *P. burmeisteri*, *P. bahiana* and *P. iheringii* as monophyletic clades, and support a close relationship between the southernmost species, in particular between the sister-taxa *P. distincta* and *P. tetraploidea*. The major distinction between northern and southern groups of *P. burmeisteri* species was not recovered by previous phylogenetic work but is in agreement with breeding call similarities within each group (single and regular versus paired and regular pulse rates, in northern and southern groups, respectively; see Pombal & Haddad 1992). The present study also offers stronger support for the distincta-tetraploidea sister relationship, which was only moderately supported (parsimony bootstrap value = 88) in the phylogenetic analysis of Faivovich *et al.* (2010). This lends additional support for the hypothesis suggesting an origin of *P. tetraploidea* from *P. distincta* (Haddad *et al.* 1994).

The origin of P. tetraploidea

The species *P. tetraploidea* was previously suggested to result from recent autopolyploidization of *P. distincta* on the basis of undergoing hybridization between species, similar mating calls and chromosomic homologies of metaphase I trivalents in triploid hybrids (Haddad *et al.* 1994). Multivalents could however reflect not the complete homology of genomes, but close relatedness be- tween genomes that are still able to pair (King 1990). Cytologic analysis could also fail in revealing an autopolyploid origin when multisomic inheritance is lost as expected after a certain time with the re-establishment of dissomic inheritance (Dewet 1980). Following the hypothesis of an autopolyploid origin of *P. tetraploidea* from *P. distincta*, it would be expected that both nuclear and mtDNA alleles of the tetraploid species cluster with *P. distincta*.

However, sequence divergence (Table 2.3) between any pair of southern (*P. iheringii*, *P. tetraploidea* and *P. distincta*) species is actually very similar for both mtDNA (5%) and nuclear data (≈0.2 - 0.6), and with sharing of nuclear alleles. We cannot therefore exclude the hypothesis that *P. tetraploidea* resulted from hybridization between *P. iheringii* and *P. distincta* followed by genome duplication (allopolyploidy). Given the high bootstrap and posterior probabilities in both concatenated and coalescent-based Bayesian species-trees supporting *P. distincta* as sister taxa of *P. tetraploidea*, it is however likely that *P. tetraploidea* resulted from genome duplication of *P. distincta*.

Considering the estimates for time of divergence between *P. tetraploidea* and *P. distincta* at about ≈0.3 million years ago, and between those and their close relative *P. iheringii* at 2.5 million years ago (Fig. 2.4), it is likely that sharing of nuclear alleles between *P. tetraploidea*, *P. distincta*, and *P. iheringii* was due to incomplete lineage sorting (see above). On the contrary, the high level of mtDNA sequence divergence among those three species may result from the lower effective population size and higher mutation rate of ND2 relative to nuclear loci, leading to much faster sorting of ancestral polymorphisms. This would also explain the lack of resolution to infer phylogenetic relationships among these three species. Because gene trees will not necessarily converge onto a similar species-tree due to stochastic variation in the coalescent process (e.g., Maddison 1997; Bachtrog *et al.* 2006), the present study exemplifies the need to use multiple loci for phylogenetic analysis of

closely related species.

Given our multilocus inference and the evidence provided by Haddad *et al.* (1994), the most likely hypothesis to explain the origin of tetraploid species should be (i) autopolyploidization from *P. distincta* or, (ii) autopolyploidization from an unknown independent lineage that shares a common ancestor with *P. distincta* (e.g., Mable & Roberts 1997; Stöck *et al.* 2006). In the latter case, replacement of the original 2n lineage by *P. tetraploidea* would not be surprising given that tetraploids may be more tolerant to different ecological conditions (for reviews, see Otto & Whitton 2000; Otto 2007). Indeed, the geographic distribution range of *P. tetraploidea* includes regions with more pronounced seasonality compared to typical BAF rainforest areas occupied by the other species in the group, with exception of *P. iheringii* (Pombal & Haddad 1992; Haddad *et al.* 1994). In the future, a genetic approach based on quantitative analyses of marker alleles transmitted in the progeny of laboratory controlled crosses could be particularly useful to better elucidate the origin of *P. tetraploidea* (see for example Chenuil *et al.* 1999).

Patterns of diversification in Phyllomedusa burmeisteri species group

MtDNA ND2 genetic diversity across the range was strikingly high with five well-supported clades showing pairwise divergences ranging from 5 - 10%. Further genetic substructuring in *P. burmeisteri* species group was suggested by well-supported sub-clades within major clades, but should be confirmed with increased sampling. The divergence between northern and southern clades started around 5 Myr. This time estimate (tMRCA) roughly coincides with that of other BAF widespread organisms (Bothrops jararaca, Grazziotin *et al.* 2006; Rhinella crucifer, Thomé *et al.* 2010). *P. distincta*, *P. tetraploidea* and *P. iheringii* may have diverged at around 2.5 Myr, while species in the northern group may have diverged more recently (1.6 Myr; Table 2.4). These levels of divergence are compatible with those for lineages of *Hypsiboas bishoffi* (2.4 Myr; J; Alexandrino, unpublished data). Diversification in these organisms should therefore be explained in the context of historical population events that occurred since the Late Tertiary (Pliocene) and throughout the Pleistocene.

Recent analyses combining phylogeographic data and paleodistribution modelling supported that endemism and genetic diversity in BAF is associated with

range stability since the Last Glacial Maximum (LGM; Carnaval & Moritz 2008; Carnaval *et al.* 2009). Two major stability areas (refugia) were identified: the Pernambuco and the Bahia refugia. The region south of São Paulo may have been unstable since the LGM and would have been recently recolonized from adjacent northern refugia (Carnaval *et al.* 2009), explaining the comparatively lower genetic diversity of two anurans observed south of the refugia areas (*H. albomarginatus*, *H. semilineatus*). Genetic diversity patterns not compatible with recent colonization have however been also recently described for reptiles, birds and other amphibians (Grazziotin *et al.* 2006; Cabanne *et al.* 2008; Fitzpatrick *et al.* 2009; Thomé *et al.* 2010; J. Alexandrino, unpublished data). In the *P. burmeisteri* group, fragmentation in northern (between mtDNA haplotypes of *P. bahiana*) and central BAF (between haplotypes of *P. burmeisteri*) would be compatible with long-term regional persistence of populations. In southern BAF, we found high genetic diversity that corresponds to divergence between *P. distincta* and *P. tetraploidea*. Because their geographic distributions both include areas in southern São Paulo, it cannot be ruled out that there were parallel southward colonizations from refugia in that region (e.g., Carnaval *et al.* 2009). The additional structure in southern BAF apparent from the mtDNA gene tree (haplotypes in localities 22, 24, 27 and 23, 25 and 26, respectively, in *P. distincta* and *P. tetraploidea*) may suggest instead longer term population persistence, but this must be confirmed with more detailed sampling.

Independent BAF palinological data suggest that forest fluctuations occurred during the Pleistocene. While the studies made in the inland region showed good support for forest fragmentation (Ledru 1993; Ledru *et al.* 1996; Behling & Lichte 1997; Behling 2002; Gouveia *et al.* 2002), data on the coastal region indicated always the presence of forest in the LGM (Saia *et al.* 2008; Ledru *et al.* 2009). This scenario of fragmentation could have impacted the diversification of the southern *P. distincta* and *P. iheringii*, as much as genetic diversity within *P. distincta* and *P. tetraploidea*, but this can only be properly examined with further studies including detailed sampling. *P. iheringii* is an ecological outlier within the treefrog group as it occurs in a southern drier area, well within the Pampas biome, and cannot be associated to the forest dynamics of the BAF. We speculate that diversification of *P. iheringii* may be associated to genetic divergence accompanied by ecological differentiation.

Phylogeographic concordance in BAF was revealed with breaks separating

major *P. burmeisteri* group clades, northern (*P. burmeisteri* and *P. bahiana*) and southern (*P. tetraploidea*, *P. distincta*, and *P. iheringii*), coinciding with those separating major clades of the treefrogs *H. faber* (Carnaval *et al.* 2009), in the whole of BAF, *H. bishoffi* (J. Alexandrino, unpublished data), *Rhinella crucifer* (Thomé *et al.* 2010), and the snake *B. jararaca* (Grazziotin *et al.* 2006), in southern São Paulo. Similar phylogeographic breaks were also revealed for BAF birds (Cabanne *et al.* 2008); and the spatial pattern is also coincident with patterns of endemism for BAF amphibians and mammals (Lynch 1979; Costa *et al.* 2000). Coincident spatial patterns of genetic and species diversity suggests common processes acting to promote biological diversification.

Geomorphological events since the Tertiary to the present day may have impacted the diversification process of BAF organisms, such as the remake of parts of the Brazilian east coast due to older tectonic and more recent neotectonic phenomena (e.g. Ribeiro, 2006; Saadi *et al*. 2002). The main break in the *P. burmeisteri* group (*P. burmeisteri*/ *P. bahiana* and *P. tetraploidea*/ *P. distincta*/ *P. iheringii*) is coincident with spatial movements of the Guapiara lineament and the Cubatão shear zone, with this one being also associated to more recent additional structure in *P. distincta* (Fig. 2.5; Saadi *et al.* 2002). The Doce River system is coincident with break between *P. burmeisteri* and *P. bahiana*. This complex and dynamic geological formation may have been a barrier for other BAF organisms (Pellegrino *et al.* 2005; Saadi *et al.* 2005; Sigrist & Carvalho 2008). The Propriá and Rio Araçuaí fault zone or Caratinga and Além Paraíba fault zone can acted together in promoting the diversification of *P. bahiana* and *P. burmeisteri* and also of awell-supported sub-clade of *P. bahiana* (Figs. 2.2 and 2.5). *P. burmeisteri* and *P. bahiana* have however an area of phenotypic intergradation that will have to be further studied at the population level to examine the spatial diversification process and species limits.

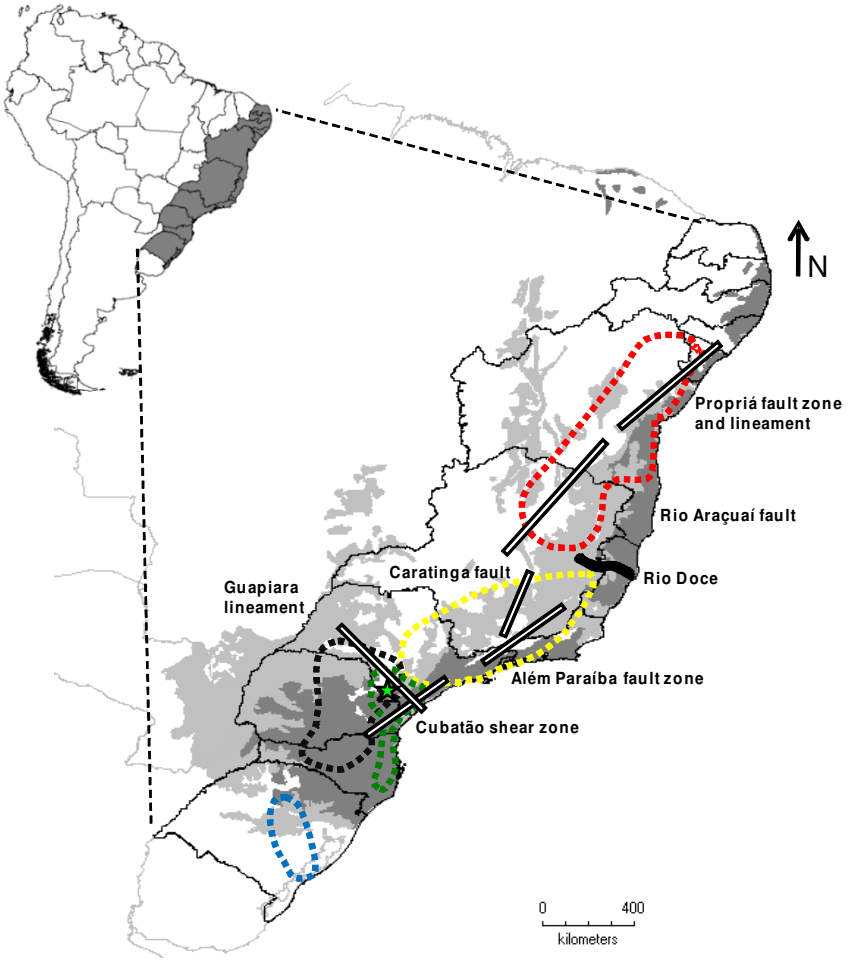

Fig. 2.5 Location of putative geographic barriers in BAF versus mtDNA clades of *Phyllomedusa burmeisteri* species group. Brazilian Atlantic Forest original cover, ombrophylous (dark gray) and semi-deciduous (light gray) are represented. Clade colours correspond to Fig 2.1 and 2.2. The green star marks the reported hybridization between *Phyllomedusa distincta* and *Phyllomedusa tetraploidea.*

Our results suggest that both the Tertiary and Quaternary were important for the diversification of species of the *P. burmeisteri* group which is in agreement with other molecular studies in BAF organisms (e.g., Lara & Patton 2000; Ribas & Miyaki 2004; Wüster *et al.* 2005; Grazziotin *et al.* 2006). While a pattern of association of phylogeographic breaks to river/ tectonic barriers is emerging in BAF, the challenge will be to undertake studies designed to distinguish between the alternative, but non-exclusive, forest refugia and barrier hypotheses to explain diversification patterns of BAF organisms.

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Capítulo 3

Padrões de distribuição da variabilidade genética e fenotípica em anfíbios com taxonomia complexa

3.1 Species limits, phylogeographic and hybridization patterns in Neotropical leaf frogs (Phyllomedusinae)[2]

3.1.1 Abstract

The taxonomy of many species is still based solely on phenotypic traits, which is often a pitfall for the understanding of evolutionary processes and historical biogeographic patterns, especially between closely related species due to either phenotypic conservatism or plasticity. Two widely distributed Neotropical leaf frogs from the *Phyllomedusa burmeisteri* species group (*P. burmeisteri* and *Phyllomedusa bahiana*) constitute a paramount example of closely related species with relatively unstable taxonomic history due to a large phenotypic variation. Herein we analysed ~ 260 individuals from 57 localities distributed across the range of the two species to contrast individual phenotypic with an integrative phylogenetic and phylogeographic multilocus approach. We aim to clarify species limits, investigate potential undocumented diversity, and examine to what extent taxonomic uncertainties could lead to misleading hypotheses on phylogeographic and interspecific hybridization patterns. Our molecular analysis supports the recognition of the two currently defined species, providing evidences for one novel and highly divergent evolutionary unit within the range of *P. burmeisteri*, which encompasses its type locality (Rio de Janeiro city). Spatial patterns of genetic and the colour of the hidden areas of the thigh was not congruent, varying considerably both within and between populations of both species. Genetic data showed signs of admixture between both species, but do not corroborate the previously inferred wide area of introgression based on the distribution of the intermediate phenotype. Our results suggest that phenotypic variation can result from local adaptations, geographic isolation, and/or evolutionary processes and, thus, cannot be used to reliably diagnose *P. burmeisteri* and *P. bahiana*. Globally, this study underscores the need of a geographical broad sampling of widespread species and the combination of molecular and phenotypic data to delineate species limits and phylogeographic patterns in species with complex taxonomy.

2 This section refers to the article: Brunes TO, Alexandrino J, Baêta D, Zina J, Haddad CFB & Sequeira F (2014) Species limits, phylogeographic and hybridization patterns in Neotropical leaf frogs (Phyllomedusinae). *Zoologica Scripta*, **43**, 586-604.

3.1.2 Introduction

Understanding the processes underlying biological diversity in the Brazilian Atlantic Forest (BAF) has been the focus of many molecular studies in past few years. Among the numerous proposed drivers of diversification, the refuge hypothesis, which is associated with the climatic-induced cyclical expansion and contraction of forests, and the tectonically mediated river dynamics are the predominant ones (Grazziotin *et al.* 2006; Thomé *et al.* 2010; Amaral *et al.* 2013). The accumulated data has shown that the origin and distribution of biodiversity in this hotspot cannot be explained by any general model, being more consistent with idiosyncratic and organism-specific response patterns to potential driving factors of diversification, generating overwhelming complexity (see review in Silva *et al.* 2012).

Despite of these studies, inadequate biodiversity inventories, taxonomic uncertainties, limited knowledge on the spatial distribution of taxa, and the absence of phylogenetic information are still serious drawbacks to undertake phylogeographic reconstructions in many taxonomic groups. The taxonomy of most Neotropical taxa or groups/species complexes is still based on phenotypic traits, which is often the cause for discordance with molecular data due to either phenotypic plasticity or morphological conservatism, often associated to highly homoplastic traits (e.g., Thomé *et al.* 2010). Indeed, molecular data have indicated that many recognized taxonomic clades are not monophyletic; while in other cases revealed deep genetic divergences within populations of species, which may often correspond to species complexes (e.g., Gehara *et al.* 2013). Moreover, the lack of integrative approaches (combining natural history with genetic, morphological, and ecological data) to clearly delimit taxonomic units associated with inconsistent criteria for species delimitation are still producing problematic taxonomies and either underestimating or overestimating the number of species (Vieites *et al.* 2009; Vences *et al.* 2013).

The Neotropical leaf frogs of the *Phyllomedusa burmeisteri* species group, with a widespread distribution throughout the BAF, constitute an excellent example of how a comprehensive phylogenetic/ phylogeographic approach linked to newly developed species delimitation methods may be used to investigate the current taxonomy of the group and to explore any undocumented species diversity. Species of the genus *Phyllomedusa* are characterized by presenting eco-physiological adaptations linked to desiccation resistance, and aposematic colour patterns of

flanks and thighs associated with skin bioactive peptides (Funkhouser 1957; Shoemaker *et al.* 1972; Faivovich *et al.* 2010; Calderon *et al.* 2011). Currently, the *P. burmeisteri* species group includes four diploid species, *P. burmeisteri*, *Phyllomedusa iheringii*, *Phyllomedusa distincta*, *P. bahiana* and one tetraploid form, *Phyllomedusa tetraploidea*. However, this group experienced a relatively unstable taxonomic history due to the large phenotypic variation and absence of alternative diagnosable phenotypic characters (see revision in Pombal & Haddad 1992). Taxonomic uncertainty has been particularly evident in the case of *P. bahiana* and *P. burmeisteri*, since the first species was synonymized to the second by B. Lutz (1950), corroborated latter by Pombal & Haddad (1992) in an extensively review, and subsequently resurrected by Silva-Filho & Juncá (2006). Indeed, Pombal & Haddad (1992) observed a northest-southest phenotypic clinal pattern between *P. bahiana* and *P. burmeisteri*, showing a tendency toward the fixation of morphotypes of these species at the extremes of each respective distribution range, with the occurrence of the two morphotypes and "intermediate" individuals in several locations of Espírito Santo and Minas Gerais states (Fig. 3.1A and 3.1B). The species revalidation was based on differences in larval morphology and vocalizations recorded in only one population (Serra de São José, Municipality of Feira de Santana, Bahia state).

More recently, Brunes *et al.* (2010) explored the phylogeny of the five morphospecies from *Phyllomedusa burmeisteri* group in a multilocus study. Mitochondrial (mtDNA) analyses recovered the monophyly of *P. bahiana*, *P. burmeisteri* and *P. iheringii*, showing that *P. distincta* and *P. tetraploidea* were paraphyletic, but not resolved the phylogenetic relationships among taxa. By contrast, both single locus nuclear (nuDNA) analysis only gave support for higher-level relationships revealing the presence of two main groups: 1) one formed by *P. bahiana* and *P. burmeisteri* (northernmost species) and other 2) by *P. distincta*, *P. tetraploidea*, and *P. iheringii* (southernmost species). This closest relationship between both the northernmost and the southernmost distributed species was confirmed by the multi-gene tree and the species tree analysis. Although the hypothesis of retention of ancestral polymorphisms was advanced to explain the lack of monophyly at the nuDNA level for the currently recognized five morphospecies, the broad scale nature of the study (i.e. small sampling of populations and loci) together with previous evidence for an extensive area of phenotypic intergradation between *P. bahiana* and *P. burmeisteri* (Pombal & Haddad 1992) precluded a more detailed

exam of alternative hypotheses to explain these results. Regarding the confusing patterns of phenotypic diversity and, in particular, the lack of comprehensive phylogeographic studies in face of the extensive distribution range of both forms (Faivovich *et al.* 2010; Brunes *et al.* 2010), we here contrasted the individual morphotype characterization with an integrative phylogenetic and phylogeographic multilocus approach (one mitochondrial and three nuclear genes) in combination with newly developed Bayesian species delimitation approaches to clarify species limits, investigate potential undocumented diversity across the ranges of *P. burmeisteri* and *P. bahiana*, and generally discuss the biogeographic patterns of diversification in BAF.

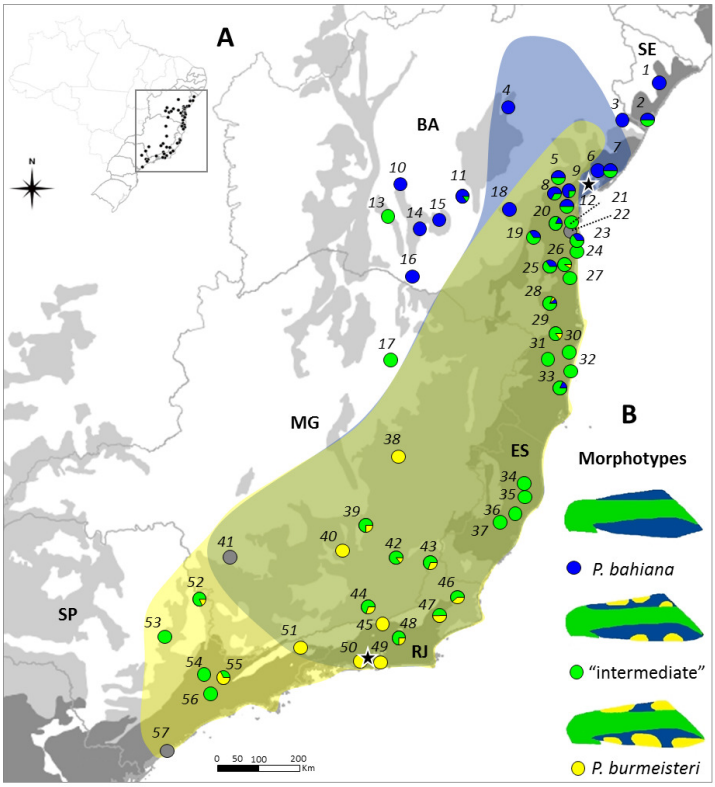

Fig. 3.1 Based on Pombal & Haddad (1992): (A) Geographic distribution of morphotypes: *Phyllomedusa bahiana* (blue), *Phyllomedusa burmeisteri* (yellow), and "intermediate" individuals (overlapped area); and (B) scheme of morphotypes following the patterns of the hidden surfaces of the thigh. Sampling localities numbered of 1 to 57 (SL; Table 3.1). Circles on map represents the proportional occurrence of each individual morphotype analysed in this study (see Table 3.1 and Table B.1). Grey circles indicates no morphotype information. Brazilian Atlantic Forest original cover: Ombrophylous (dark grey) and Semideciduous/Deciduous (light grey) forests are represented. Stars indicate type localities. Brazilian states: SE, Sergipe; BA, Bahia; MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo.

3.1.3 Material and methods

Sampling

We collected individuals of *Phyllomedusa burmeisteri*, *P. bahiana*, and their morphological intermediates from all over the species ranges (Pombal & Haddad

1992). We obtained a total of 269 individual's tissue samples from 57 localities (see Sampling localities, SL) by field collecting trips from November of 2010 to February of 2012 and by several tissue donations from different Brazilian herpetological collections (see Acknowledgments and Table B.1). Most of the occurrence points were georeferenced with GPS coordinates and the remaining were based on the coordinates from the closest town (Table 3.1 and Fig. 3.1A). Both species were collected in places ranging from ~ 5 to 1031 meters above sea level. Samples consisted of liver, toe clips, or muscle preserved in 100% ethanol. All collected individuals were fixed in 10% formalin solution for a week, preserved in 70% ethyl alcohol, and deposited in three Brazilian Herpetological collections: Célio F. B. Haddad amphibian collection (CFBH), Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista, Rio Claro, SP; Coleção Zoológica da Universidade Estadual do Sudoeste da Bahia (CZUESB), Jequié, BA; and, Coleção Herpetológica da Universidade Federal de Juiz de Fora (CHUFJF), Departamento de Zoologia, MG, Brazil (vouchers from Juiz de Fora locality).

Table 3.1 Columns indicate sampling locality information, sample sizes, haplotype number, mitochondrial clade and morphotype proportional values of *Phyllomedusa bahiana* and *Phyllomedusa burmeisteri*. State abbreviations (ST): SE, Sergipe; BA, Bahia; MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo. Morphotype classification: U = *Phyllomedusa bahiana*, I = "intermediate", and S = *Phyllomedusa burmeisteri*.

SL	Locality	ST	Longitude	Latitude	N	Haplotypes	mtDNA			Morphotypes	
							BAH	BUR	BUR-RJ	U	I
1	Areia Branca	SE	-37.3153	-10.7578	1	1	1.0	-	-	1.0	-
2	Indiaroba	SE	-37.5131	-11.5194	2	2,3	1.0	-	-	0.5	0.5
3	Acajutiba	BA	-38.0576	-11.5724	4	2,4,5	1.0	-	-	1.0	-
4	Itaitú	BA	-40.4004	-11.3274	9	2,4,6,7	1.0	-	-	1.0	-
5	Castro Alves	BA	-39.3880	-12.7239	2	2	1.0	-	-	0.5	0.5
6	Cachoeira	BA	-38.6200	-12.6231	8	2,4,8	1.0	-	-	0.9	0.1
7	Dias d'Ávila	BA	-38.2990	-12.6028	2	9,10	1.0	-	-	0.5	0.5
8	Amargosa	BA	-39.3852	-13.0058	3	2,4,11	1.0	-	-	0.7	0.3
9	Santo Antônio de Jesus	BA	-39.1548	-12.9970	4	2,11	1.0	-	-	0.75	0.25
10	Contendas do Sincorá	BA	-42.6831	-12.9164	1	10	1.0	-	-	1.0	-
11	Mucugê	BA	-41.4056	-13.1213	10	2,12,13	1.0	-	-	0.9	0.1
12	Valença	BA	-39.2229	-13.3241	2	14	1.0	-	-	0.5	0.5
13	Riacho Santana	BA	-42.9352	-13.5948	1	15	1.0	-	-	-	1.0
14	Caetité*	BA	-42.2816	-13.8323	3	15,16	1.0	-	-	1.0	-
15	Livramento de Nª Senhora	BA	-41.8454	-13.6498	1	15	1.0	-	-	1.0	-
16	Jacaraci	BA	-42.4404	-14.8475	1	15	1.0	-	-	1.0	-
17	Grão Mogol	MG	-42.9024	-16.5909	4	17,18	1.0	-	-	-	1.0
18	Maracás	BA	-40.4308	-13.4411	12	2,4,14,19-21	1.0	-	-	1.0	-
19	Jequié	BA	-39.9376	-13.9834	14	4,7,11,22,23	1.0	-	-	0.3	0.7
20	Gandu	BA	-39.4401	-13.7348	11	11.14.24-26	1.0	-	-	0.2	0.8

SL	Locality	ST	Longitude	Latitude	N	Haplotypes	mtDNA			Morphotypes	
							BAH	BUR	BUR-RJ	U	I
21	Ituberá	BA	-39.1542	-13.7318	1	7	1.0	-	-	-	1.0
22	Igrapiúna	BA	-39.1710	-13.8212	8	11,27	1.0	-	-	-	-
23	Camamu	BA	-39.1082	-13.9704	3	11,28	1.0	-	-	0.3	0.7
24	Itacaré	BA	-39.0279	-14.2918	2	29	1.0	-	-	-	1.0
25	Aurelino Leal	BA	-39.5395	-14.5734	6	11,29,30	1.0	-	-	0.3	0.7
26	Uruçuca	BA	-39.2844	-14.5931	8	11,29,31	1.0	-	-	-	0.9
27	Ilhéus*	BA	-39.1724	-14.7957	12	11,32,33	1.0	-	-	-	1.0
28	Camacan*	BA	-39.0587	-15.4164	14	11,29,33-39	1.0	-	-	0.2	0.7
29	Itapebi	BA	-39.0541	-16.0045	5	40,41,42	-	1.0	-	-	0.8
30	Porto Seguro	BA	-39.1805	-16.4081	2	40,43	-	1.0	-	-	1.0
31	Itabela	BA	-39.6781	-16.5653	2	44,45	-	1.0	-	-	1.0
32	Caraíva	BA	-39.1478	-16.8081	1	29	1.0	-	-	-	1.0
33	Prado	BA	-39.3976	-17.1606	6	40,41,46,47	-	1.0	-	0.2	0.8
34	Sooretama	ES	-40.0978	-19.1969	2	48	-	1.0	-	-	1.0
35	Linhares	ES	-40.0722	-19.3911	5	49-52	-	1.0	-	-	1.0
36	Aracruz	ES	-40.2733	-19.8203	8	11,29,52-54	0.5	0.5	-	-	1.0
37	Santa Teresa	ES	-40.6050	-19.9525	2	55,56	-	1.0	-	-	1.0
38	São João Evangelista	MG	-42.7494	-18.5461	1	57	-	1.0	-	-	-
39	Catas Altas	MG	-43.4378	-20.0562	4	58- 60	-	1.0	-	-	0.75
40	Congonhas	MG	-43.8759	-20.4883	1	61	-	1.0	-	-	-
41	Furnas	MG	-46.2459	-20.6564	1	62	-	1.0	-	-	-
42	Viçosa	MG	-42.7050	-20.7050	7	62-64	-	1.0	-	-	0.8
43	Carangola	MG	-42.0836	-20.7986	11	56,63,65	-	1.0	-	-	0.7
44	Juiz de Fora	MG	-43.3696	-21.7325	13	61,63,66,67	-	1.0	-	-	0.7
45	Três Rios	RJ	-43.2159	-22.1124	1	66	-	1.0	-	-	-
46	Campos dos Goytacazes	RJ	-41.4906	-21.5276	5	68-70	-	-	1.0	-	0.6
47	Santa Maria Madalena	RJ	-41.9384	-21.8811	4	68,70,71	-	-	1.0	-	0.5
48	Cachoeiras de Macacu*	RJ	-42.7491	-22.4257	7	72,73	-	-	1.0	-	0.7
49	Niterói	RJ	-43.1045	-22.8798	1	73	-	-	1.0	-	-
0.5	Rio de Janeiro	RJ	-43.5315	-22.8278	1	74	-	-	1.0	-	-
51	Queluz	SP	-44.7739	-22.5369	1	75	-	1.0	-	-	-
52	São José do Rio Pardo	SP	-46.8727	-21.5493	5	62,76	-	1.0	-	-	0.8
53	Rio Claro	SP	-47.6755	-22.3472	1	62	-	1.0	-	-	1.0
54	Jundiaí*	SP	-46.8172	-23.1647	11	77,78	-	1.0	-	-	1.0
55	Nazaré Paulista	SP	-46.4191	-23.2373	3	77,79	-	1.0	-	0.3	0.7
56	“Grande” São Paulo*	SP	-46.6361	-23.5475	4	77,80	-	1.0	-	-	1.0
57	Iguape	SP	-47.5414	-24.6981	3	77,81	-	1.0	-	-	-

* Localities with different sample sizes in genetic and phenotypic analysis.

Qualitative phenotypic variation

Qualitative phenotypic characterization was focused on the pattern of the hidden surface of the thigh, as proposed by Pombal & Haddad (1992): *P. burmeisteri* presenting yellowish rounded spots on a bluish background (S); *P. bahiana* with a coloured blue uniform without the presence of rounded blotches (U); and the

"intermediate" individuals with smaller and fewer yellowish rounded blotches (I) (Fig. 3.1B). Assuming that the pattern observed for "intermediate" individuals could be under or overestimated when compared with that of *P. burmeisteri*, we adopted a conservative approach and considered only individuals with three yellowish rounded blotches in both thighs as *P. burmeisteri*, whilst individuals with less than three were considerate intermediate.

Laboratory procedures

Genetic analyses included a total of 269 individuals of *P. bahiana*, *P. burmeisteri*, and their intermediates. With exception of *P. tetraploidea*, we used three *P. distincta* and two *P. iheringii* individuals as representatives of the remaining species of the group, and *P. boliviana* as outgroup. The data include some sequence information from previous studies (Faivovich *et al.* 2010; Brunes *et al.* 2010) available in GenBank (see Table B.1). Whole genomic DNA was obtained through tissue samples digested in lysis buffer and Proteinase K and using QIA Quick DNEasy columns (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. We sequenced a fragment of the mitochondrial NADH dehydrogenase subunit 2 gene (ND2) and three nuclear genes: i) a segment of exon 2 and intron 2 of the cellular myelocytomatosis (C-myc2), ii) a segment of exon 2 of chemokine receptor 4 (CXCR4), and iii) a segment of β-fibrinogen intron 7 (β-fibint7).

PCR amplification and sequencing protocols for ND2 and β-fibint7 were performed following Brunes *et al.* (2010). For C-myc2 and CXCR4 PCR amplification, we specifically designed two pairs of internal primers: CMYC-PHYF (5'-TCCAAAGTTGGCTCTCAATGC-3') and CMYC-PHYR (5'-GAGTCTCTGCCCTAAACTATTC-3'); CXCR4-PHYF (5'-GTCCAGGACCATGACTGACAAG-3') and CXCR4-PHYR2 (5'-TTCGGTGATGGCGATCCACTTG-3'), respectively. PCR reactions were performed in 20µl reaction volume containing 10 µL Qiagen PCR Master Mix, ~50 ng of genomic DNA and 0.2 µM each primer. Cmyc2 amplification consisted of a pre-denaturing step of 3 min at 92 ºC, followed by 40 cycles of a denaturing step of 30 s at 92 ºC, annealing at 58 ºC for 30 s and extension at 72 ºC for 90 s. The final extension was accomplished at 72 ºC for 5 min. CXCR4 amplification conditions consisted of an initial denaturation at 95 ºC for 15 min, followed by a touchdown program with 10

cycles of 95 °C for 30 s, 56 °C to 52 °C for 30s, decreasing 0.5 ºC in each cycle, and 72 °C for 45 s, followed by 32 cycles of 95 °C for 30 s, 52 °C for 30s and 72 °C for 45 s with a final extension at 60 °C for 30 min. Sequencing was performed from enzymatically purified PCR products in the ABI Prism BigDye Terminator Cycle sequencing protocol in an ABI PRISM 3130 XL Genetic Analyser and by the Macrogen Corporation sequencing facility (http://www.macrogen.com). All electropherograms were checked for errors and assembled contiguous sequences using the CodonCode Aligner (CodonCode Corporation, Dedham, MA). Alignments were edited in BIOEDIT v.7.0.5.2 (Hall 1999) and correct by eye.

Sequence variation

Heterozygous insertions or deletions (indels) of nuclear DNA sequences were identified with the mutation detection tool in CodonCode Aligner, and through eye inspection of chromatograms. Next, we used the PHASE software v.2.1 (Stephens *et al*. 2001) with the assistance of SeqPHASE (Flot 2010) to resolve the haplotype phases. Multiple independent runs were performed for each gene with different seeds for the random-number generator and 1.0 x 10^6 iterations with the default values. Haplotype estimation was checked through consistency analysis across runs. We selected the haplotype reconstructions above P = 0.90 or the most likely phased. Additionally, we detected multiple-base indels in two nuclear loci and reduced it to a single evolutionary step following Brunes *et al*. (2010). The presence of recombination events were evaluated through the Difference of Sums of Squares (DSS) test implemented in TOPALi v.2.5 (Milne *et al*. 2004) using all phased haplotypes, a window size of 70 bp, steps 10 bp-long, and 100 bootstrap repetitions. Alignment Transformation Environment (ALTER; Glez-Peña *et al*. 2010) was used to transform datasets to the input formats of down-the-line software used for analyses. Polymorphism values including segregation sites (S), number of haplotypes (h), haplotype diversity (Hd), and population mutation parameter (Θ) were calculated for mitochondrial and nuclear data.

Phylogenetic analyses and haplotype networks

We reconstructed a phylogenetic tree under Bayesian Inference (BI) for mitochondrial data and maximum parsimony (MP) haplotype genealogies for nuclear sequences.

To select the best-fit partitioning schemes we used the PartitionFinder v.1.0.1 (Lanfear *et al.* 2012) and the models of molecular evolution were selected in jModelTest v.0.1.1 (Posada 2008) under the Akaike information criterion (AIC; Akaike 1974), following Posada & Buckley (2004). BI analyses were performed in MrBayes version v.3.2 (Ronquist & Huelsenbeck 2003) through two replicate searches using the stop rule command fixed with a standard deviation of 0.01 (stop value) sampling every 1000 generations. Four MCMC (Markov chain Monte Carlo) were run simultaneously in each analysis. To analyze the nuclear data we used the haplotype genealogy method to avoid the presence of reticulation and provides a "clear" scenario to sets of closely related species. They were produced starting from a MP tree though DNAPARS available in PHYLIP v.3.69 package (Felsenstein 2005) following results of Salzburger *et al.* (2011). The best parsimony tree was converted in a haplotype genealogy in a beta version of the Haploviewer (http://www.cibiv.at/~greg/haploviewer). Detailed information of locality, voucher number and sequenced genes for individuals is shown in Table B.1.

Pairwise distance and neutrality tests

For mtDNA we calculated the uncorrected pairwise distances (*p*-uncorrected) between the major mtDNA clades, between sub-clades and within each sub-clades. For ncDNA, the same genetic distance was calculated between major groups as revealed by STRUCTURE analysis (see Results). Estimates of 95% confidence intervals were generated with 10,000 coalescent simulations. To evaluate if mitochondrial sub-clades and nuclear groups present deviations from the neutral theory we computed Tajima's D (Tajima 1989) and R2 test (Ramos-Onsins & Rozas 2002) and significance of values were checked also through 10,000 coalescent simulations. All analyses were performed in DNAsp v.5.10 (Librado & Rozas 2009).

Species delimitation, species tree, and divergence times estimates

To investigate species delimitation, we performed several multilocus analyses with different methods: i) a Bayesian clustering method (STRUCTURE, Pritchard *et al*. 2000) that not require *a-priori* assignment of individuals to clusters; and three methods that assume *a-priori* assignment of individuals to clusters: ii) a genetic distance matrix network (POFAD, Joly & Bruneau 2006); and two additional methods

based on the coalescent theory: iii) *BEAST (Heled & Drummond 2010); and iv) Bayesian species delimitation (BPP, Rannala & Yang 2003; Yang & Rannala 2010). The *BEAST was used to estimate a species tree and the time of the most recent common ancestor (tMRCA). The resulting species tree was used to evaluate the limits of the putative species using the software BPP (e.g. Tsai & Carstens 2013). Regarding the no accommodation of recent admixture in both *BEAST and BPP, we used individuals only from allopatric areas. This 'allopatric dataset' comprises eight *P. bahiana* individuals from Sergipe and north Bahia state (BAH clade), nine *P. burmeisteri* individuals from south São Paulo state (BUR clade), and ten *P. burmeisteri* individuals from Rio de Janeiro state (BUR-RJ clade) (see details Table B.1). For comparative purposes, we used the same allopatric dataset for POFAD analysis.

Genetic distances between individuals were calculated for each nuDNA fragment in MEGA v.5.2.2 (Tamura *et al*. 2011) and combined into a multilocus distance matrix in POFAD v.1.03. Both standardized and non-standardized matrices were produced in order to evaluate the possible understatement of attribution of the same weight for all matrices. The multilocus distance network was visualized in Splits Tree v.4.12.3 (Huson & Bryant 2006) via NeighborNet method. To access the genetic assignment to population clusters within the northern distributed species from the P. burmeisteri group we used the Bayesian model-based algorithm implemented in the program STRUCTURE v.2.3.4. To that purpose, the sequence information for at least two nuDNA fragments per individual were converted to allele frequency data through the program xmfa2struct (available at: http://www.xavierdidelot.xtreemhost.com/clonalframe.htm) for a total of 114 individuals from 41 localities (see details in Table B.1). Analysis was performed under the admixture ancestry model, with five independent runs for each K ranging from 1 to 10. The first 1 x 10^5 MCMC iterations were discarding as burn-in and the next 25x104 repetitions were counted. The best K value was found via the on-line program Structure Harvester v.0.6.93 (Earl & VonHoldt 2012) to monitor the estimated log posterior probability of the data (ln Pr (X/K)) (Pritchard *et al*. 2000), and estimate the second-order rate of change of the likelihood function (ΔK) (Evanno *et al.* 2005). Finally, the results of the five independent runs were assembled in the program CLUMPP v.1.1.2 (Jakobsson & Rosenberg 2007) and checked for biologically meaningful population clusters.

To estimate a species tree of the northern distributed species of the *P. burmeisteri* group we used two datasets (mtDNA and nuDNA combined and only nuDNA), whilst diversification times (tMRCA) were estimated only with the mtDNA and nuDNA combined dataset. For that, we used the Bayesian Markov chain Monte Carlo method for the multispecies coalescent method implemented in the software *BEAST v.1.8.0. We added the sequence information of *P. boliviana*, *P. distincta*, and *P. iheringii* (see details in Table B.1) to the 'allopatric dataset' as explained above. Diversification times were based on a ND2 mutation rate (0.00957 mutations/site/million years; see Crawford 2003), primarily estimated for *Eleutherodactylus* species (Anura: Leptodactylidae). However, given the lack of fossil data and independent calibration points, this mutation rate has been previously applied in several anuran phylogeographic studies with appropriate prior adjustments (Carnaval & Bates, 2007; Brunes *et al*. 2010; Thomé *et al*. 2010; Nuñez *et al*. 2011). We recognize that this type of non-specific approach is not free of drawbacks and could be a potential source of inference error (see Edwards & Beerli 2000). Thus, diversification times should be interpreted carefully. We performed preliminary analysis (data not shown) searching for the best model of molecular clock rate variation (Strict Clock or Relaxed Clock: Uncorrelated Lognormal) for each fragment and for the best tree model prior ("Yule" or "Birth and Death") for the data. Performance and accuracy of analysis were checked in the software Tracer v.1.5 (https://web.archive.org/web/20130926165911/http://beast.bio.ed.ac.uk/Tracer). The final analysis was performed with a speciation "Birth and Death" process as tree prior and relaxed clock with an uncorrelated lognormal distribution for ND2 and C-myc2, and a strict clock for β-fibint7 and CXCR4 in an independent run sampling every 10,000 generations for 100 million generations with 10% of burn-in. The final tree was obtained in the TreeAnnotator v.1.7.4 (https://web.archive.org/web/20130806231432/http://beast.bio.ed.ac.uk/TreeAnnotator) and previewed in FigTree v.1.1.2 (https://web.archive.org/web/20140221095451/http://beast.bio.ed.ac.uk/FigTree).

Species delimitation analysis was conducted using the Bayesian species delimitation method (BPP). This method accommodates the species phylogeny as well as lineage sorting due to ancestral polymorphism. We also performed two analysis with different datasets as in *BEAST (see above). To test the species limits of the northern distributed species of *P. burmeisteri* group we incorporate *P. distincta*,

P. iheringii, and *P. boliviana* information and used the species tree estimate by *BEAST as rooted guide tree. Considering the low number of taxa and its close relationships (see Leaché & Fujita 2010), we adopted conservative values for the gamma prior (G) in population size (θ = 2, 2000) and for the divergence time parameter for the root in the species tree (τ0 = 2, 2000). These values means relatively small ancestral population sizes and shallow divergences among species, while the other divergence time parameters are assigned the Dirichlet prior (Yang & Rannala 2010: equation 2). Each rjMCMC analysis was run at least twice to confirm consistency between runs using 200 000 generations and 20 000 as burn-in.

3.1.4 Results

Qualitative phenotypic variation

Of the total of 269 individuals analysed, 233 were phenotypically characterized following the pattern of the hidden surfaces of the thigh proposed by Pombal & Haddad (1992). Figure 3.1A shows morphotype distributions and Table 3.1 presents their proportional values in each locality. In general, the “intermediate” morphotype was more frequent (60%) and showed a widespread distribution from north to south of BAF. The *P. burmeisteri* morphotype was the least frequent (11%), although it showed a moderate distribution along the BAF. The uniform blue morphotype (*P. bahiana*) was observed in 29% of the specimens and showed a more restricted distribution mainly in north of Bahia state, with the exception of three specimens located in southeast of Bahia state (SL 28 and 33; Fig. 3.1A). This last morphotype showed a particular distribution pattern mostly occupying some patches of BAF besides the continuous ombrophilus area. The sympatric occurrence of the three morphotypes was observed only in one locality (SL 28; Fig. 3.1A) with the prevalence of the intermediate one. We highlighted in Fig. 3.1A morphotype localities represented by only a single specimen and therefore the morphotype assigned to these localities should be interpreted with caution.

Sequence variation

We analysed one mitochondrial gene for the 267 individuals and three nuclear genes for a representative subsample of *P. bahiana* and *P. burmeisteri* individuals (Table B.1). We obtained a mitochondrial fragment (ND2) of 993 base pairs (bp) which

revealed a total of 81 haplotypes with 203 segregating sites. For the nuclear fragments, individuals of each species were grouped according to the STRUCTURE results (Fig. 3.5B). Overall, we analysed 250 gene copies of 609bp for CXCR4, 122 gene copies of 600-604bp for β-fibint7, and 144 gene copies of 345-348bp for C-myc2. The number of segregating sites ranged from 29 in C-myc2 to 65 in β-fibint7, haplotypes numbers from 31 in CXCR4 to 71 in β-fibint7 and the theta parameter (Θ) ranged from 0.86% in CXCR4 to 2.1% in β-fibint7 (Table 3.2). The DSS test did not detect recombination in any of the fragments.

Phylogenetic analyses and haplotype genealogies

The best-fit partitioning schemes for the mtDNA fragment favoured the use of distinct evolutionary models for each codon position (AIC: 7134.03). Models of nucleotide substitution used were: TrN+I+G (p-inv = 0.3330, gamma shape = 0.4770) for the 1st position, TrN+I (p-inv = 0.8000) for the 2nd position, and GTR+G (gamma shape = 3.3920) for 3rd position. BI mtDNA analysis revealed the presence of three highly divergent well-supported clades (Fig. 3.2). The first one supported the monophyly of *P. bahiana* comprising haplotypes distributed from Sergipe state up to Espírito Santo state in which *P. burmeisteri* haplotypes also occur in the same locality (SL 36; Table 3.1; Fig. 3.5A). The *P. bahiana* clade (BAH) also presented two sub-clades, Sub-clade A (Sc-A) and Sc-B, with high and moderate support, respectively. While Sc-A comprises haplotypes found in more deciduous and xeric (Caatinga) areas, Sc-B consists mostly of haplotypes from coastal and moister areas. Four localities in the transition between sub-clades areas shared haplotypes from Sc-A and Sc-B (SL 8, 9, 19, and 20) (Table 3.1 and Fig. 3.2). The second main clade occupies the largest portion of the *P. burmeisteri* range (BUR), with the exception of the majority of the haplotypes from Rio de Janeiro state (SL 46–50) that form their own divergent clade (BUR-RJ) (Figs. 3.2 and 3.5A). The BUR clade also showed well-supported sub-clades: Sc-C including southern Bahia haplotypes; Sub-clade D (Sc-D) with haplotypes in the Espírito Santo, Minas Gerais, and São Paulo states, and haplotypes from southern Bahia (SL 29, 31, and 33); and, Sub-clade E (Sc-E) presenting one unique haplotype from the Rio de Janeiro state (SL 45), and exclusive haplotypes from the Minas Gerais and São Paulo states (SL 42, 43, 44, 56, and 57) (Table 3.1 and Fig. 3.2).

Table 3.2 Fragment information, summary statistics, genetic distances (p-uncorrected) and neutrality tests within the main mitochondrial clades and nuclear groups (see STRUCTURE results) of *Phyllomedusa bahiana* and *Phyllomedusa burmeisteri*.

Fragment	Clade/Group	Length	Polymorphism						Neutrality test's	
			N	S	h	% Hd	% θ	*p* [95% C.I.]	Tajima's D	R^2
ND2	All	993	267	203	81	95	3.3	-	-	-
	BAH		154	55	39	89	1	-	-	-
	Sc-A		71	27	19	88	0.5	0.27 [0.05-0.7]	-1.6291 ns	0.0491ns
	Sc-B		83	22	20	71	0.4	0.16 [0.02-0.4]	-1.9207**	0.0335**
	BUR		95	71	35	95	1.4	-	-	-
	Sc-C		11	5	5	62	0.17	0.1 [0-0.28]	-1.7910**	0.1311*
	Sc-D		44	41	22	93	0.95	0.6 [0.2-1.5]	-1.2118 ns	0.0695 ns
	Sc-E		38	12	7	75	0.3	0.4 [0.1-1.1]	- 1.9257 ns	0.1910 ns
	BUR-RJ		18	17	7	86	0.5	0.53 [0.1-1.3]	0.2478 ns	0.1549 ns
CXCR4	All	609	250	32	31	84	0.86	-	-	-
	P. bahiana		72	20	10	53	0.68	0.26 [0.03-0.72]	-1.8315*	0.0468*
	P. burmeisteri		178	22	24	78	0.63	0.41[0.1-1.07]	-0.9326 ns	0.0567 ns
β-fibint7	All	600-604	122	65	71	98	2.1	-	-	-
	P. bahiana		44	34	28	96	1.3	1.4 [0.3-2]	0.0118 ns	0.1185 ns
	P. burmeisteri		78	42	47	97	1.4	0.5 [0.16-1.2]	-1.3593 ns	0.0592 ns
C-myc2	All	345-348	144	29	62	96	1.5	-	-	-
	P. bahiana		64	21	31	94	1.3	0.3 [0.05-0.7]	-1.3918 ns	0.0606 ns
	P. burmeisteri		88	14	34	93	0.8	0.15 [0.01-0.36]	-1.5226 ns	0.0454 ns

* $p < 0.05$ ** $p < 0.01$ ns = no significance

Single locus nuclear genealogies did not recover any of the three clades presented in the mtDNA analysis (Fig. 3.3). However, two out of the three fragments (CXCR4 and C-myc2) showed a strong tendency to separate two haplogroups: one formed by *P. bahiana* individuals (BAH) and another with *P. burmeisteri* individuals. All nuclear genealogies were congruent in presenting shared polymorphism, as six haplotypes in β-fibint7, four in both CXCR4 and C-myc2 are shared by individuals belonging to two or three different mtDNA-defined groups (BAH, BUR, and BUR-RJ). In both C-myc2 and CXCR4, some haplotypes are clustered in the BAH group, but their corresponding mtDNA haplotype belongs to BUR group, and vice versa (SL 31, 33 and 36). Throughout the visual inspection of the network topologies, only BAH group from CXCR4 exhibits a more pronounced pattern reflected by a star-like topology, which is indicative of population demographic expansion.

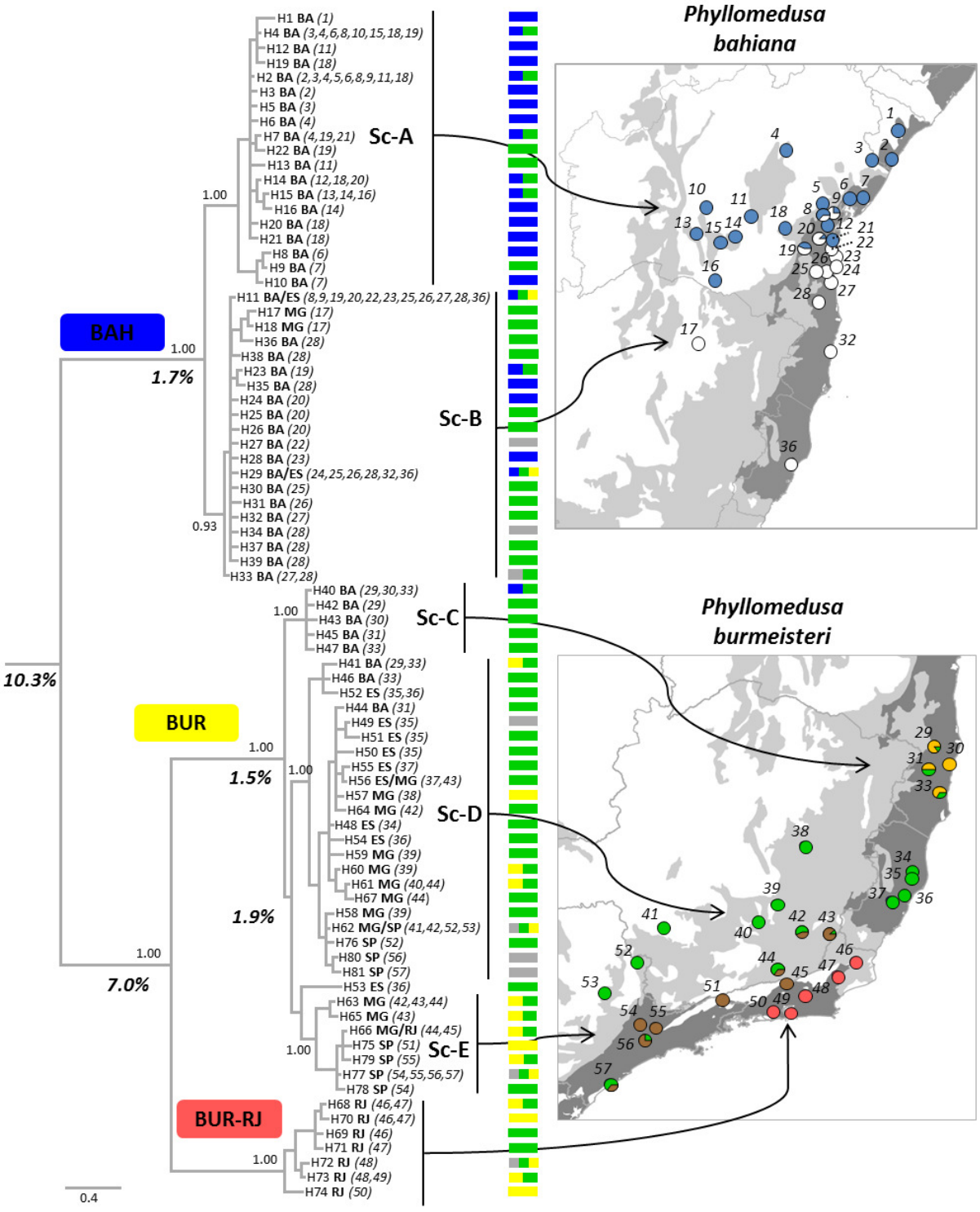

Fig. 3.2 Mitochondrial gene tree (ND2) derived from Bayesian analysis of *Phyllomedusa bahiana* and *Phyllomedusa burmeisteri* and sub-clades (Sc-A-E) distribution on map. Terminal names indicate haplotype number, initial of states and locality code (see Fig. 1 and Table 1). Bayesian posterior probabilities (over 90%) are given near the branches. Percentages indicate *p*-uncorrected values between clades. Arrows indicate the respective sub-clade distribution. Brazilian Atlantic Forest original cover: Ombrophylous (dark grey) and Semideciduous/Deciduous (light grey) forests are represented.

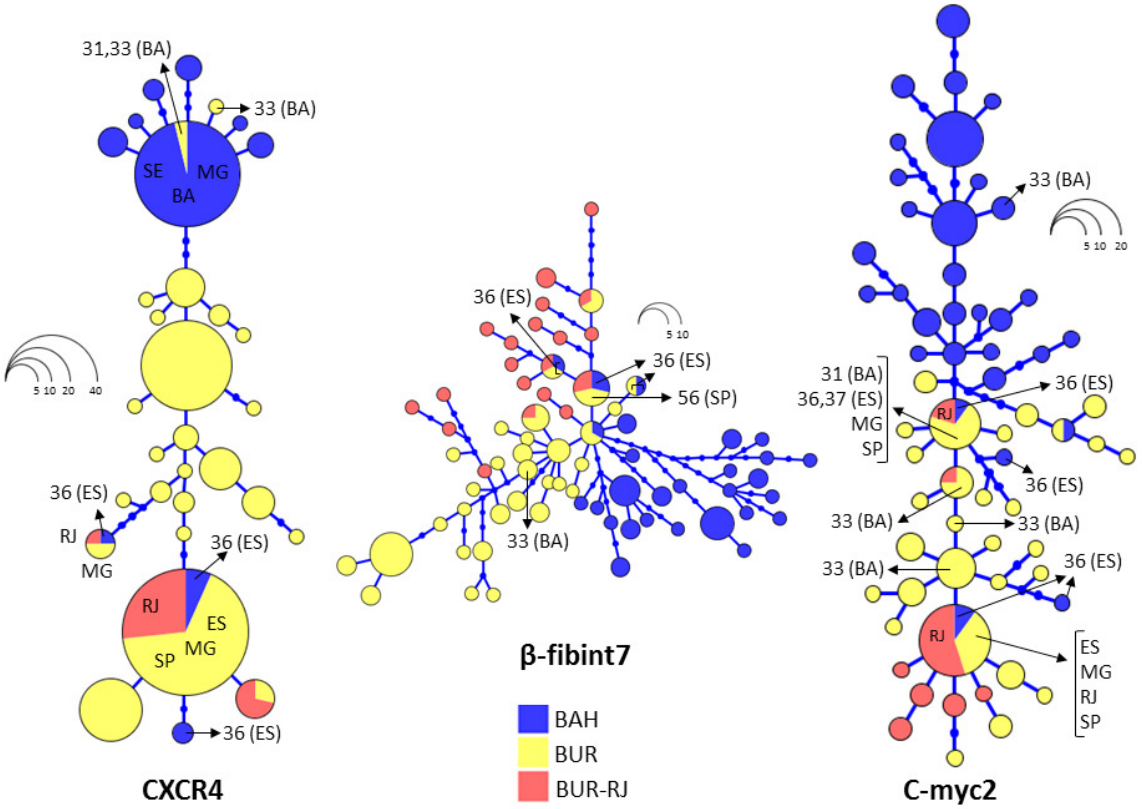

Fig. 3.3 Nuclear haplotype genealogies converted from parsimony trees in the software Haploviewer of *Phyllomedusa bahiana* and *Phyllomedusa burmeisteri*. The circle area of each haplotype is proportional to its frequency. Mutations are edges. Colours represents the three major mitochondrial lineages (see Fig. 3.2). Arrows indicate sampling localities (SL) and Brazilian state, respectively (see Table 3.1 and Fig. 3.1).

Pairwise distance and neutrality tests

Mitochondrial uncorrected pairwise distances values between clades and sub-clades are presented in Figure 3.2. While a pairwise distance of about 10% was found between *P. burmeisteri* and *P. bahiana*, an unexpected high level (7%) was found between the two *P. burmeisteri* clades (BUR and BUR-RJ). All sub-clades presented similar distance values ranging from 1.5 to 1.9%. In general, intra-clade distances were higher within *P. burmeisteri* than in *P. bahiana* (Table 3.2). Neutrality tests applied to the mtDNA dataset were both in agreement showing that individuals from Sc-B from (*P. bahiana*) and Sc-C (*P. burmeisteri*) might be undergoing a process of demographic expansion. Both tests performed with data from the nuclear CXCR4 fragment showed that *P. bahiana* could be undergoing expansion (Table 3.2).

Genetic distance network and population assignment analyses

The multilocus distance network was not congruent with the results from mtDNA.

Instead of presenting three divergent groups, the POFAD analysis revealed two main groups: one clustering individuals from *P. bahiana* (BAH) and another with *P. burmeisteri* individuals from both BUR-RJ and BUR distribution (Fig. 3.4). There is a strong tendency to separate BUR from BUR-RJ individuals with some transitional individuals between both lineages: five from BUR (SL 54 and 56) and two from BUR-RJ (SL 46 and 48). Individuals from the Jundiaí locality (SL 54) in São Paulo state, clustered with both the divergent BUR group and transitional individuals.

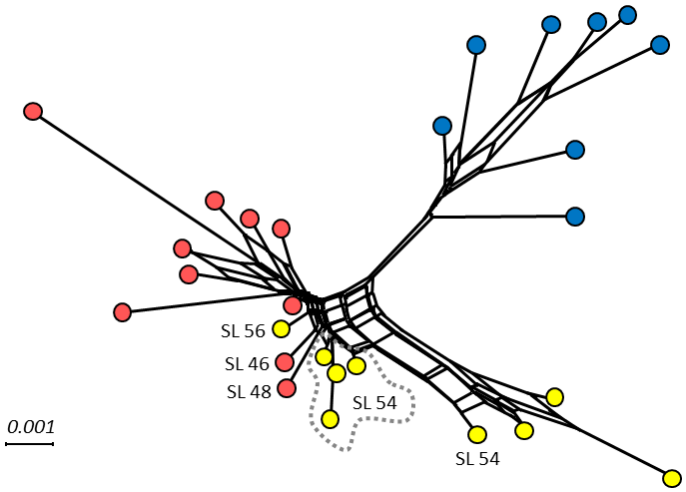

Fig. 3.4 Multilocus genetic distance network of *Phyllomedusa bahiana* and *Phyllomedusa burmeisteri* based on three nuclear fragments (CXCR4, β-fibint7, C-myc2) of the allopatric dataset. Circles were coloured following the three major mitochondrial groups (see Fig. 3.2 and 3.5). Sampling localities numbers (SL) are in Table 3.1 and Fig. 3.1.

The Bayesian clustering analysis, according to the distribution of Ln Pr (X/K) and ΔK, recovered the nuclear DNA structure of the northern species of the *P. burmeisteri* group as K = 2, corresponding to *P. bahiana* individuals (BAH) from *P. burmeisteri* (BUR and BUR-RJ) (Fig. 3.5B and Fig. B.1). The geographic break between these two clusters is located in southern of Bahia state, roughly coincident with the Jequitinhonha river with the exception of individuals of *P. bahiana* from SL 17 that penetrated into the interior part of northern of Minas Gerais state. STRUCTURE results also evidenced the presence of some putative hybrid individuals in Itabela and Prado localities (SL 31 and 33, respectively), showing in addition that all individuals from SL 36 (according to mtDNA four of which are clustered in *P. bahiana* group) belong to *P. burmeisteri* at nuclear level.

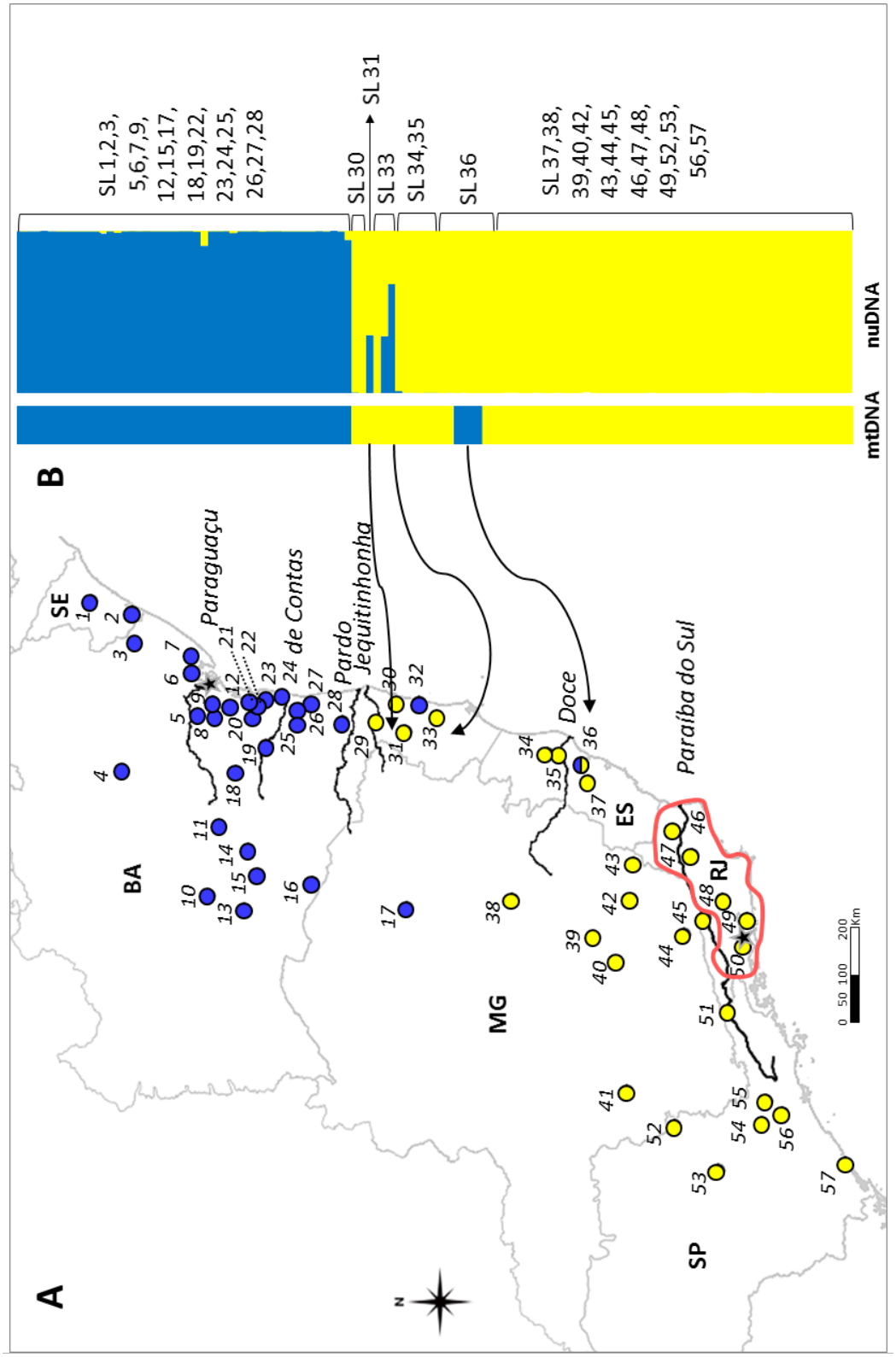

Fig. 3.5 Mitochondrial DNA distribution (A) and STRUCTURE results based in nuDNA allele frequencies for K=2 (B) of Phyllomedusa bahiana (blue) and Phyllomedusa burmeisteri (yellow). Individuals are represented as bars, with colours representing the proportion of assignment. Bars on the left represent the respective mitochondrial main clades. Surrounds area in red represents the BUR RJ clade (Fig. 3.2). Sampling localities (SL) follow Fig. 3.1 and Table 3.1. Major rivers of the Brazilian East Atlantic Basin are indicated. Stars indicate type localities.

Species delimitation, species trees and divergence times estimates

Both species trees showed a high support for two main clades; one grouping the southern species *P. distincta* (P. dis) and *P. iheringii* (P. ihe) and another with the northern species: *P. bahiana* (BAH), *P. burmeisteri* (individuals from BUR and BUR-RJ lineages). The species tree also supported the split between BUR and BUR-RJ lineages as sister-taxa (Fig. 3.6A and Fig. B.2A). The Bayesian coalescent estimate for tMRCA was ~ 2.9 Myr (95% CI; 1.4–4.4) for the split between *P. bahiana* and *P. burmeisteri* (BUR and BUR-RJ) and ~ 1.3 Myr (95% CI; 0.4–2.4) for the split between BUR and BUR-RJ lineages (Fig. 3.6A). The BPP species delimitation analyses, either combining mtDNA and nuDNA (Fig. 3.6B) or using only nuDNA data (Fig. B.2B), were strongly congruent and showed posterior probabilities equal or higher 0.99 in all nodes tested. Overall, the five units inferred in the BPP analysis (*P. distincta*, *P. iheringii*, BAH, BUR, and BUR-RJ) were highly supported as species or as candidate species in the case of BUR lineage.

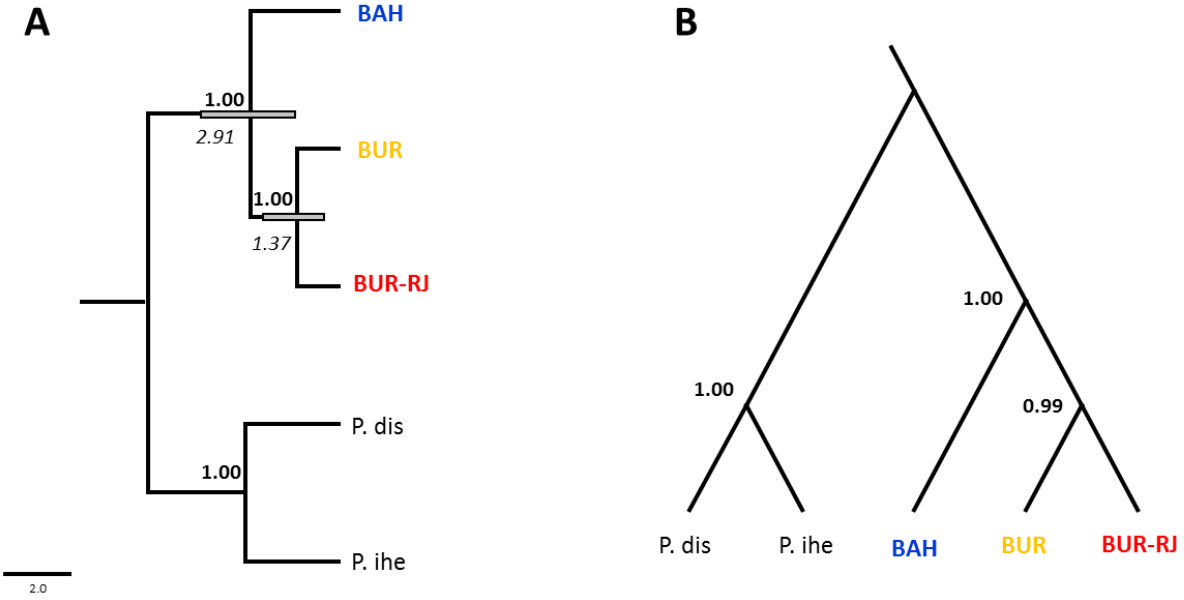

Fig. 3.6 Coalescent multilocus analysis combining mitochondrial and nuclear DNA information of partial *Phyllomedusa burmeisteri* group: (A) Species tree in *BEAST; (B) Species delimitation in BPP. Posterior probabilities are shown above of the branches and the mean times to the most recent common ancestor (tMRCA) are bellow of the branches. The node grey bars represent the 95% confidence intervals. P. dis, *Phyllomedusa distincta*; P. ihe, *Phyllomedusa iheringii*.

3.1.5 Discussion

Phylogeny, genetic structure and cryptic diversity

The phylogenetic analysis of a mtDNA gene fragment (ND2) showed two deeply divergent (p-distance = 10%) and highly supported mtDNA clades, which exhibit a

geographic cohesiveness broadly coincident with the previously inferred ranges of both *P. bahiana* and *P. burmeisteri* in BAF (Fig. 3.2). Our results showed also deep mitochondrial structure within each of the two main clades, corresponding broadly to previous results uncovered by Brunes *et al.* (2010). A new finding though was the observation of two highly divergent (p-uncorrected = 7%) and well-supported monophyletic clades (BUR and BUR-RJ) within *P. burmeisteri*. This novel clade recovered by our current analysis shows geographical cohesiveness, corresponding to a previously unsampled geographic area of the Rio de Janeiro State (Fig. 3.2), including the city of Rio de Janeiro which is the type-locality of *P. burmeisteri* (Fig. 3.5A, Boulenger 1882).

The nuclear gene genealogies did not fully recover the same population structure observed in the mitochondrial tree (Fig. 3.3). Discordance between nuclear and mitochondrial markers is expected in analyses of closely related species (e.g. Degnan & Rosenberg 2009). However, both single and multilocus networks showed a general trend towards uncovering two groups concordant with the *P. bahiana* and *P. burmeisteri* (BAH and BUR/BUR-RJ) mtDNA clades. This result is corroborated by our Bayesian cluster assignment which consistently recovered two clusters that largely correspond to the two deeply diverged mtDNA clades, therefore suggesting the signature of a major evolutionary genetic break. Bayesian assignment of individuals showed signs of admixture at the nuclear level between both clusters in southern Bahia state (SL 31 and 33). Interestingly, no signs of nuclear admixture were found in SL 36 (Espírito Santo state), the single locality where mtDNA haplotypes of *P. burmeisteri* and *P. bahiana* co-occur (Fig. 3.5A and B). Although we cannot exclude that retention of ancestral polymorphism may explain the pattern of sharing haplotypes in our single locus nuclear genealogies (Fig. 3.3), the pattern of mtDNA distribution together with nuclear Bayesian assignment results may provide additional evidence for past or current gene flow between these two evolutionary genetic units (see detailed discussion below).

Within *P. burmeisteri*, only the multilocus distance network was roughly congruent with the occurrence of two clusters of haplotypes that correspond to two divergent mtDNA clades (BUR and BUR-RJ). Possible explanations for the lack of reciprocal monophyly in the nuclear genealogies includes the possibility of gene flow, since BUR-RJ clade is geographically surrounded by the BUR clade, incomplete lineage sorting due to recent diversification as previously described for the *P.*

burmeisteri species group (Brunes *et al.* 2010), and the longer times of neutral coalescence of nuclear DNA compared to the mitochondrial genome (e.g., Zink & Barrowclough 2008). Indeed, the Bayesian coalescent estimates of divergence time (tMRCA) were twice as old for the split between *P. bahiana* and *P. burmeisteri* clades (~ 2.8 Myr) than for the split between BUR and BUR-RJ clades (~ 1.3 Myr; Fig. 3.4A).

The Bayesian clustering analyses did not recover any genetic substructure within the *P. burmeisteri* clade, which could additionally be explained by the reduced power of reliably inferring the optimal number of clusters due to low number of loci here analysed (e.g. Thomé *et al.* 2012). In recent years, many studies have used nuclear sequence data coupled with assignment methods for revealing cryptic genetic structure and investigate species delimitation using similar or slightly higher numbers of loci (3–6) in groups with complex taxonomy (Leaché & Fujita 2010; Thomé *et al.* 2012; Fusinatto *et al.* 2013; Welton *et al.* 2013). Although the performance of these methods has been varied with different levels of genetic divergence, in most cases they have successfully detected relevant genetic structure. The power to detect genetic structure with a reduced number of loci in Bayesian model-based assignment methods must also depend on the markers used, adequate sampling of individuals, as well as the species evolutionary history (Yang *et al.* 2005; Hausdorf & Hennig 2010). Here, the use of relatively conserved nuclear markers did not allow the detection of further genetic structure within the major clades (i.e. finer level structure). The use of fast-evolving markers such as microsatellites (Brunes *et al.* 2013) may be instrumental in this respect to increase the power of detecting genetic clustering at shallower lineage divergences.

Patterns of genetic and phenotypic variation

An overall discordance between the genetic data and the variation in colour pattern on the hidden surfaces of the thigh (morphotype) was observed in this study. The morphotypes previously assigned to *P. bahiana* and *P. burmeisteri* (pure morphotypes), were reported to occur within the distribution range of each species albeit a few cases of sympatry (Pombal & Haddad 1992). Our results showed that the intermediate morphotype is clearly the most frequently observed across the ranges of both species, co-occurring with pure morphotypes at several localities (this includes the two phenotypically indistinguishable evolutionary units of *P. burmeisteri*, BUR and

BUR-RJ). Most of individuals assigned to each of the two identified nuclear clusters with a probability of approximately 100% (q = 1.0) therefore exhibit the intermediate morphotype, suggesting that the colour patterns of the hidden surfaces of the thigh is not a diagnostic character between *P. bahiana* and *P. burmeisteri*.

Discordance between phenotypic traits and patterns of genetic divergence is a widespread phenomenon among amphibians (Ohmer *et al.* 2009; Rodríguez *et al.* 2012; Brusa *et al.* 2013; Bruschi *et al.* 2013; but see *Agalychnis callidryas* in Robertson *et al.* 2009). Colour polymorphism can arise and be maintained by the action of isolated or combined evolutionary processes such as natural and sexual selection (Sandoval & Nosil 2005; Richards & Knowles 2007), genetic drift (Hoffman *et al.* 2006; Abbott *et al.* 2008), gene flow (Slatkin 1985), and anti-predator strategy and conspecific communication (Toledo & Haddad 2009; Rudh & Qvarnström 2013). The occurrence of an intermediate morphotype has been previously interpreted as the result of extensive gene flow between *P. bahiana* and *P. burmeisteri* (Pombal & Haddad 1992). Based on this hypothesis, we expected that high levels of gene flow combined with limited localized selection have contributed to the widespread geographic distribution of the intermediate morphotype. However, the signature of extensive admixture between both species was not detected in this study, since either individuals with an assignment probability lower than 70% (q < 0.7; three) or with 100% (q = 1.0) but with introgressed mtDNA (four individuals classified as *P. burmeisteri* with nuclear DNA have mtDNA from *P. bahiana*), are relatively few and restricted to some localities in southern Bahia and Espírito Santo. Alternatively, the spatial pattern and frequency of the intermediate morphotype may result from natural selection acting on defensive strategies. In species of *Phyllomedusa*, the colour patterns of the hidden surfaces of flanks and/or thighs (bright uniform colours or mixed with yellow spots or irregular stripes) have been interpreted as aposematic, given that these patterns are displayed when individuals move on tree branches stimulated by the presence of potential predators (Toledo & Haddad 2009; Toledo *et al.* 2011; but see Rudh & Qvarnström 2013). The most important point of the theory explaining aposematism (Müller 1879) is that populations of distinct species should converge into one similar aposematic phenotype because in this way the predator's behaviour will be reinforced to avoid that specific colouration pattern (e.g. Endler 1982; Harmon *et al.* 2005). In this situation, selection is expected to favour the most conspicuous and common colour pattern (directional selection). So, it is possible that

the highest frequency and widespread distribution of the intermediate morphotype reflects the most successfully pattern of coloration as a defensive mechanism. While our sample of a single phenotypic trait and the use of neutral markers cannot conveniently address this hypothesis, intra-populational variability in colour patterns present in both species deserves further study, with particular emphasis on the role of sexual selection, ecological traits, and behavioural strategies.

Species tree and estimates of species delimitation

Estimates of coalescent species trees are efficient to accommodate inconsistencies between gene genealogies due to incomplete lineage sorting in recently diversified populations (Heled & Drummond 2010). In this study we used the Bayesian coalescent species tree implemented in *BEAST to contrast the phylogenetic relationships inferred by mtDNA and nuclear data when separately analysed. To avoid the confounding effect of contact zones between adjacent groups, since we detected potential gene flow, we used only individuals from geographic regions distant from the contact zones ("allopatric" dataset) to estimate a species tree and to determine species limits between *P. bahiana* and *P. burmeisteri*. The species trees (*BEAST) recovered a sister-taxa relationship between *P. bahiana* and *P. burmeisteri*, and two sister-clades within *P. burmeisteri* (BUR and BUR-RJ), with high posterior probability support for all nodes. The Bayesian species delimitation method (BPP) consistently supported the three distinct evolutionary units (BAH, BUR, and BUR-RJ). In both cases, results were therefore concordant with the mtDNA tree topology, irrespectively of using only the nuclear DNA dataset (Fig. B.2A and B) or combining mtDNA and nuclear datasets (Fig. 3.6A and 6B).

There is a growing body of research on exploring methods for testing species delimitation, since inferring boundaries between evolutionary lineages has become paramount to estimate and describe species diversity, particularly in cases of complex taxonomy (Leaché & Fujita 2010; Fusinatto *et al.* 2013; Satler *et al.* 2013; Sousa-Neves *et al.* 2013). However, these methods are not free of drawbacks (e.g. testing a priori delimited units based on a single guide tree), and the outcomes have greatly varied among studies. While several authors have presented taxonomic recommendations (Niemiller *et al.* 2012; Fusinatto *et al.* 2013) or even have described new taxonomic entities (Leaché & Fujita 2010; Satler *et al.* 2013), others

have recognized only possible taxonomic implications without presenting formal recommendation (Setiadi *et al.* 2011; Camargo *et al.* 2012; Welton *et al.* 2013).

In the case of *P. burmeisteri*, there are several challenges for the taxonomic significance of the novel evolutionary unit unveiled in this study (BUR), since its delimitation is not fully congruent across different types of markers and methods. By contrast, our molecular analyses are congruent in supporting an independent evolutionary trajectory of the two current recognized species, *P. bahiana* and *P. burmeisteri*. Moreover, it is notable that the putative existence of a second taxa within *P. burmeisteri* (BUR-RJ clade) would imply a drastic reduction of the current known range of this species, since type-locality of *P. burmeisteri* is from Rio de Janeiro city (Fig. 3.5A). Indeed, individuals from BUR clade occupies the largest portion of the known range assigned currently to *P. burmeisteri*, whereas individuals from the BUR-RJ clade are restricted to the Rio de Janeiro state. Notwithstanding, the taxonomic status of these two species and particularly of this novel evolutionary unit would benefit from an integrative approach including other sources of information, such as reproductive aspects (e.g. advertisement calls) related with pre-zygotic isolation.

Biogeography and spatio-temporal patterns of diversification

Patterns of diversification in the *Phyllomedusa burmeisteri* species group have been recently addressed by Brunes *et al.* (2010). Following these authors, species from this group were originated between ~ 0.3 and 2.5 Myr, spanning the late Pliocene and Pleistocene. Here, using geographically-widespread sampling, the split time between *P. bahiana* and *P. burmeisteri* was estimated to occur at ~ 2.9 Myr (95% CI; 1.4–4.4). Although this time interval is within the confidence interval estimated by Brunes *et al.* (2010), the more ancient split time inferred here probably reflects the addition of one more nuclear DNA locus (CXCR4) and more individuals, especially those from the novel and highly divergent evolutionary unit uncovered within the *P. burmeisteri* clade (BUR-RJ). Following theoretical expectations, population subdivision can increase coalescence time between genes and inflate estimates of population divergence time (e.g., Edwards & Beerli 2000). In addition, it has been demonstrated that both increasing number of individuals and loci is an important factor affecting the accuracy of estimates, especially in systems with shallow histories (McCormack *et al.* 2009). The second major split was estimated to occur between the two sister-evolutionary units of *P. burmeisteri* (BUR and BUR-RJ) at around 1.3 Myr

(95% CI; 0.4¬–2.4). These results corroborate previous findings on temporal patterns of diversification in many BAF organisms, suggesting that their populations were affected by historical events that occurred during the Pliocene and Pleistocene periods (e.g. Pellegrino *et al.* 2005; Grazziotin *et al.* 2006; Thomé *et al.* 2010; Amaro *et al.* 2012).

Our results from mitochondrial and nuclear analysis are concordant in defining the geographic boundary between *P. bahiana* and *P. burmeisteri* in the vicinity of the river Jequitinhonha, which disagree with the previously southernmost range limit (Doce River) inferred by Brunes *et al.* (2010). However, results presented herein showed the co-occurrence of *P. bahiana* and *P. burmeisteri* mtDNA haplotypes in Espírito Santo state (SL 36) close to the Doce River (Fig. 3.5A). The individuals bearing the two *P. bahiana* haplotypes (H11 and H29; four individuals) found in Espírito Santo state were assigned to *P. burmeisteri* on the basis of nuclear markers, suggesting that the presence of both haplotypes in this region may be a remnant of a wider distribution of *P. bahiana* in the past. The presence of foreign mtDNA as a wake of past hybridization is a widespread phenomenon among animals, including several amphibians (Babik *et al.* 2003; Sequeira *et al.* 2005; but see Toews & Brelsford 2012). Indeed, mtDNA usually tends to be more sensitive to stochastic processes than nuclear loci due to lower effective population size, maternal inheritance, and lack of recombination. Accordingly, these results may reflect a combination of distinct episodes of secondary contact between *P. bahiana* and *P. burmeisteri*: an older hybridization event in the Espírito Santo region, and a more recent secondary contact in southern Bahia.

The association of phylogeographic breaks to river barriers has already been mentioned for various BAF organisms including amphibians, lizards, and birds (Pellegrino *et al.* 2005; Thomé *et al.* 2010; Maldonado-Coelho 2012). It is well-documented that Plio-Pleistocene climatic oscillations induced sea level fluctuations along the Brazilian coast that have contributed to change the coastal plains of rivers across time and were likely responsible for recurrent episodes of isolation and secondary contact between populations (Dominguez 2009, and references therein). There is a growing body of literature documenting that Pleistocene climatic oscillations have promoted cyclic changes on environmental conditions and concomitant alterations of habitats in BAF that profoundly impacted the evolutionary history of several species (e.g. Cabanne *et al.* 2007; Carnaval & Bates 2007; Valdez

& D'Elía 2013). Regarding potential range shifts of *P. bahiana* and *P. burmeisteri*, the combined effects of complex river dynamics and Pleistocene induced extinction-recolonization events could explain both general phylogeographic patterns and more specifically the geographic location of the two putative secondary contact zones.

Regarding the ample debate about Pleistocene refuges in BAF (e.g. Carnaval *et al.* 2009), one of the most striking findings was the detection of two evolutionary units (BUR and BUR-RJ) within the range of *P. burmeisteri*, whose split spanned the Pleistocene (~ 1.3 myr). While BUR exhibits a wide distribution range, BUR-RJ is restricted to a small area coincident with the Serra do Mar in the Rio de Janeiro state, being roughly delimited by the Paraíba do Sul River (Fig. 3.5A). This area has been referred as a small putative Pleistocene refugium, exhibiting high levels of endemic species (Rocha *et al.* 2004) and evolutionary units (Pellegrino *et al.* 2005; Amaral *et al.* 2013; Gehara *et al.* 2013), which may corroborate by several assumptions for the existence of a large refugium in the southeast of Brazil based on various methodologies (see Porto *et al.* 2013, and references therein).

Additional evidence supporting a southeastern refugium hypothesis are provided by the high levels of genetic substructuring within *P. burmeisteri* (BUR clade), as suggested by the detection of three well-supported and geographically delimited mtDNA subclades (Sc-C, Sc-D, and Sc-E; p-uncorrected = 1.5–1.9%; Fig. 3.2). This hypothesis is however difficult to corroborate given the little evidence of population size changes. Indeed, Tajima's D and R2 only detected consistently signatures of population expansion for the northernmost subclade Sc-C found in southern Bahia. Our results may favour instead the hypothesis that there have been at least several local refugia allowing the species to persist through multiple climatic cycles. Some authors suggested that some BAF regions such as the foothills of mountain ranges and valleys of large rivers could have provided suitable habitats for the persistence of organisms during adverse climatic periods of the Pleistocene (see Maldonado-Coelho 2012, and references therein).

The species *P. bahiana* also revealed high levels of genetic substructure, exhibiting two well-supported and geographically delimited mtDNA subclades (Sc-A and Sc-B; p-uncorrected = 1.7%; Fig. 3.2). Both Tajima's D and R2 consistently detected signatures of population expansion only for Sc-B. This sub-clade is mostly distributed along the coast following the ombrophylous forest-type and meets with *P. burmeisteri* in southern Bahia. Our results suggest that this area of sympatry

between *P. bahiana* and *P. burmeisteri* in southern of Bahia could have resulted from the southern expansion of Sc-B and the northern expansion of Sc-C, establishing a relatively recent secondary contact zone. Regarding Sc-A, it occurs in a large area containing small patches of BAF surrounded by open Caatinga vegetation, with no signs of further substructure from the rest of the subclade range.

Taken together, our results suggest that a mosaic of habitats in heterogeneous landscapes could be of major importance for species of this group to persist though changing environmental conditions. While this hypothesis deserves further detailed molecular studies, including extended fine-scale sample sizes and the use of faster-evolving markers to examine routes and levels of gene flow among populations, the eco-physiological features (uricotelism, lipids glands and wiping behaviour) present in phyllomedusine frogs (see Faivovich *et al.* 2010, and references therein) could favoured the persistence of populations during extreme environmental changes, both those that putatively occurred during the Pleistocene and those that may be occurring in the present.

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3.2 Characterization of 12 polymorphic microsatellite markers for the Neotropical leaf-frog *Phyllomedusa burmeisteri* (Hylidae: Phyllomedusinae) and cross-species amplification[3]

3.2.1 Abstract

Twelve polymorphic tetranucleotide microsatellite loci were isolated and characterized for the leaf-frog *Phyllomedusa burmeisteri*, an endemic species of the Brazilian Atlantic forest. These loci were screened in 25 individuals from 2 populations of the Minas Gerais state (Carangola and Juiz de Fora). The number of alleles per locus ranged from 3 to 16 (mean, Na = 8). Observed and expected heterozygosities ranged from 0.25 to 0.92 and 0.56 to 0.92, respectively. Evidence for both the presence of null alleles and Hardy–Weinberg equilibrium deviations were found in loci Phybu4, Phybu17, and Phybu21. Genotypic disequilibrium for each pair of loci across populations was not significant. Cross-species amplification was successful for 11 of the 12 developed loci for the sister-species, *P. bahiana*. These microsatellites will be important for future fine-scale population structure analyses. Key words: Microsatellites; 454 sequencing; Leaf-frog; *Phyllomedusa burmeisteri*; Brazilian Atlantic forest; Cross-amplification.

3.2.2 Introduction

The Brazilian Atlantic forest (BAF) is one of 25 biodiversity hotspots given priority for conservation (Myers *et al.* 2000). According to recent estimates (Morellato & Haddad 2000; Ribeiro *et al.* 2009), only 7 to 11% of the original BAF remains today. Two of the most important factors causing this massive devastation are associated with the high level of industrialization and human population density (approximately 110 million people) present in this region. These factors have contributed to either the decline or the extinction of several organisms (Silvano & Segalla 2005).

The leaf-frog *Phyllomedusa burmeisteri* together with *P. tetraploidea*, *P. bahiana*, *P. distincta*, and *P. iheringii* belongs to the *P. burmeisteri* group, endemic to the Atlantic forest. In a recent study, Brunes *et al*. (2010) showed unexpected levels of mitochondrial divergence (5.3 to10.2%) within this species group. Using a

3 This section refers to the published article: Brunes TO, van de Vliet MS, Lopes S, Alexandrino J, Haddad CFB, Sequeira F (2013) Characterization of polymorphic microsatellite markers for the Neotropical leaf-frog *Phyllomedusa burmeisteri* and cross-species amplification. *Genetics and Molecular Research* **12**, 242-247.

multilocus approach, these authors also revealed a deep phylogenetic break near the São Paulo state between 2 main geographically coherent clusters that likely diverged approximately 5 mya: the first comprising the northerly distributed species, *P. bahiana* and *P. burmeisteri*, and the other formed by the southerly distributed species, *P. distincta*, *P. tetraploidea* and *P. iheringii*. Additionally, Brunes *et al.* (2010) have uncovered high levels of intraspecific genetic variability in all species, particularly within *P. burmeisteri* (average = 1.83%). This may be explained by the wide distribution of *P. burmeisteri* (approximately 426.510 km^2) combined with high levels of habitat fragmentation in the BAF (Pombal & Haddad 1992). Herein, we used *P. burmeisteri* as the target species to develop variable markers for future fine-scale studies, in order to better understand the role of habitat degradation on the current patterns of population genetic structure and conservation status. Cross-species amplification was performed with the sister-species *P. bahiana*, which may be important for the future genetic study of the extensive phenotypic cline between these 2 species (Pombal & Haddad 1992).

3.2.2 Material and Methods

We used 2 methodologies to construct microsatellite libraries: (1) we started with a traditional enrichment method following Van de Vliet *et al.* (2009), and (2) we used the 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries (GenoScreen, Lille, France; www.genoscreen.fr). For the 1st method, genomic DNA was isolated from a single leaf-frog using a standard phenol-chloroform extraction protocol (Sambrook *et al.* 1989). Oligonucleotide repeat probes were 5′-biotinylated using biotin-16-ddUTP (Roche, Amadora, Portugal) and DNA fragments were hybridized to a single biotinylated $(ATAG)_8$ probe or were hybridized to a mixture of biotinylated probes: $(CA)_{15}$, $(GA)_{15}$, and $(CAGA)_8$. Finally, isolated DNA fragments were screened for microsatellites by oligonucleotide probes, which were labeled with the ECL3′-oligolabelling and detection system (Amersham Pharmacia, Amersham, UK).

For the 2nd method, DNA was isolated from a gene pool of 12 individuals using Qiagen (Hilden, Germany) DNeasy spin columns. Microsatellite libraries were developed through 454 GS-FLX Titanium pyrosequencing (GenoScreen) as described in Malausa *et al.* (2011). Briefly, 1 µg DNA was enriched for AG, AC, AAC, AAG, AGG, ACG, ACAT, and ATCT repeat motifs. Amplified polymerase chain

reaction (PCR) products were purified, quantified, and then GsFLX libraries were developed following manufacturer protocols (Roche) and sequenced on a GsFLX-PTP. Finally, the redundant sequences were filtered in the QDD program (Meglécz *et al.* 2010), resulting in a final data set appropriate for primer design.

Preferably, primers were designed for tetranucleotide microsatellites with 1 unique repeat type in the DNA fragment. We favored this kind of repeat to reduce the presence of stutter bands and to improve the scoring process. A total of 36 primers (12 and 24 for the 1st and 2nd methods, respectively) were selected for further testing of amplification success and polymorphism. In addition, to prevent inconsistent amplifications in the multiplex PCR, primer pairs were tested for primer-dimer and intramolecular hairpin formation using the Autodimer software (Vallone & Butler 2004).

We estimated polymorphism levels by genotyping a total of 25 individuals from 2 populations of the Minas Gerais state: Juiz de Fora (N = 12; S 21.7325°, W 43.3696°) and Carangola (N = 13; S 20.7986°. W 42.0836°) using Qiagen Multiplex PCR Master Mix 2X. For each locus, the forward primer was 5′-labeled with a fluorescent dye (VIC, PET, FAM or NED). PCR amplifications were performed in a 10-µL reaction volume containing 5µL Qiagen PCR Master Mix, 1 µL primer mix, (0.025 µM forward primer, 0.25 µM reverse primer, and fluorescent dye of each primer), 3 µL RNase-free water and approximately 100 ng DNA template.

PCR cycling conditions consisted of an initial denaturation at 95ºC for 15 min; followed by a touchdown program with 13 cycles of 95°C for 30 s, 64°C to 58°C for 60s, decreasing 0.5ºC in each cycle, and 72°C for 40 s; followed by 19 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 40s, and by 8 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 40s; with a final extension at 60°C for 30 min. From the PCR-diluted products, we used 1 µL in combination with 10 µL deionized formamide and 0.2 µL internal size standard (Genescan-500 LIZ, ABI). Fragment size was determined on an ABI prism 3130XL capillary sequencer. Fragments were scored and binned using GeneMapper v3.7 (Applied Biosystems, Foster City, CA, USA).

We used MICROCHECKER v2.2.3 (Van Oosterhout *et al.* 2004) to check for the presence of null alleles at each locus (99% confidence level). GENEPOP on the web v3.4 (Raymond & Rousset 1995) was used to test for Hardy–Weinberg and linkage disequilibria for each locus and across populations. We used the Markov chain method with 10,000 dememorization steps and 1000 batches of 10,000

interactions per batch. All results for multiple tests were adjusted using Bonferroni correction (Rice 1989). Cross-species amplification was performed in the sister-species *P. bahiana*, using 4 individuals from different populations. Multiplex PCR was conducted using the same conditions as those used for *P. burmeisteri*.

3.2.3 Results and Discussion

Twenty-four (9 and 15 for the 1st and the 2nd methods, respectively) loci did not amplify, resulted in unexpected product sizes or ambiguous multiple bands using multiplex PCR. The remaining 12 were polymorphic loci, presented the correct product size and after several trials were arranged in 4 multiplex PCRs. Nine loci originated from the GenoScreen method (multiplex PCR: I, II, and III), and 3 originated from traditional enrichment (multiplex PCR: IV; Table 3.3).

Table 3.3 summarizes the characteristics of the 12 primer pairs of polymorphic loci derived from the leaf-frog *P. burmeisteri*. Evidence of null alleles was only statistically significant for loci Phybu4, Phybu17, and Phybu21 in the Carangola population. We found no evidence for large allele dropouts and stuttering. The number of alleles per locus ranged from 3 to 16 (mean; Na = 8) and allele sizes ranged from 124 to 329 bp. Observed and expected heterozygosities ranged from 0.25 to 0.92 and 0.56 to 0.92, respectively (Table 3.4). Genotypic disequilibrium tests for each pair of loci in each population were not significant. The loci Phybu4, Phybu17, and Phybu21 were not in Hardy–Weinberg equilibrium in the Carangola population, which likely resulted from the reduced sample size associated with the presence of null alleles.

Eleven of the 12 developed loci were successfully amplified in *P. bahiana* with the expected size range, and 8 were polymorphic (Table 3.4). Due to the high cross-amplification success observed in this study, amplification of these markers should be further tested in other species of the *P. burmeisteri* group (*P. distincta*, *P. tetraploidea*, and *P. iheringii*). These microsatellite loci may be especially important for future analyses of fine-scale population structure in *P. burmeisteri* and also for genetic characterization of the extensive morphological cline between *P. burmeisteri* and *P. bahiana*.

Table 3.3 Polymorphism at 12 tetranucleotide microsatellite markers developed for *Phyllomedusa burmeisteri*.

Locus	Primer sequence (5' – 3')	Repeat motif	Multiplex reaction	GenBank accession No.
Phybu3	F:VIC-CTCAGGGCAGCGTAAAATA R:AAACCCGACAGACTCCATTG	$(TCTA)_{13}$	I	JQ890059
Phybu4	F:VIC-CCACCTCCATGAAGACTGC R:AGGTATATTAACCATGCAGGTAGC	$(TAGA)_{14}$	II	JQ890060
Phybu5	F:NED-TCATGGATGTTACAGGCCAA R:GGCCTTTGGGGAACTGTATC	$(ATCT)_{14}$	II	JQ890061
Phybu6	F:NED-CTCCCTCCCTTCTCAGCAGT R:TCGACCTTGACAAACTATGTAACTATG	$(ATCT)_{15}$	I	JQ890062
Phybu7	F:PET-CTGCTGTGGAGACAAGCACT R: AGACCTGTCTATAGTGTAGGGCTAAT	$(CTAT)_{16}$	I	JQ890063
Phybu11	F:VIC-AACACGCCATGTGAACAAAC R:ATCTGGCCCTAACAGAATGC	$(GATA)_{17}$	III	JQ890064
Phybu17	F:6-FAM-TTCTGGCTCTGTGCAAAATG R:TCGGCTTTAGGGGTTAATGA	$(CTAT)_{12}$	III	JQ890065
Phybu18	F:6-FAM-CTTAAACATTTGAAACAGACATACAAG R:CACATGCTCCATAGTAGCAAAGA	$(TAGA)_{13}$	II	JQ890066
Phybu21	F:NED-CTGAGCTGATACATTTCATGGC R:TCTGACCCTAACAGAATGCCT	$(GATA)_{14}$	III	JQ890067
Phybu26	F:6-FAM-TCCAGAAGTGGAAAACACCAAACACA R:CAGAAAGACTCCCCCATGCCCT	$(GATA)_{9}$	IV	JQ890068
Phybu27	F:PET-CAGAACGAGGAGTTTCAAGGGC R: AACAATGCATGGATGATTTAAAGGGA	$(TCTA)_{17}$	IV	JQ890069
Phybu28	F:VIC-AGCTGTTATAGGAACGGGGTTGT R:TTGTGTGCTGCTGTTATTGTTATGTG	$(ATCT)_{21}$	IV	JQ890070

For each locus, we list the primer pair with the respective tail, the repeat motif from the original sequence, the multiplex reaction, and the GenBank accession number. F = forward and R = reverse sequences.

Table 3.4 Polymorphism statistics at 12 tetranucleotide microsatellite markers in two populations of *Phyllomedusa burmeisteri* and cross-species amplification success in *Phyllomedusa bahiana*.

Locus	Size range (pb)	Carangola (n = 13)		Juíz de Fora (n = 12)	
		Na	***Ho*/*He***	Na	***Ho*/*He***
Phybu3	229-266	10	0.833/0.854	5	0.750/0.739
Phybu4	141-193	11	0.500/0.843 *	6	0.666/0.715
Phybu5	215-305	16	0.923/0.917	5	0.625/0.726
Phybu6	129-169	10	0.846/0.861	7	0.909/0.760
Phybu7	180-262	9	0.833/0.819	6	0.750/0.680
Phybu11	243-311	11	0.846/0.864	7	0.833/0.788
Phybu17	241-329	8	0.250/0.819 *	7	0.818/0.810
Phybu18	124-168	9	0.923/0.775	3	0.636/0.558
Phybu21	202-270	10	0.250/0.871 *	7	0.800/0.815
Phybu26	125-203	10	0.750/0.826	6	0.916/0.767
Phybu27	153-201	8	0.692/0.846	7	0.916/0.781
Phybu28	187-257	10	0.769/0.828	9	0.600/0.855

Allele size length in base pairs (bp) and number of alleles (Na) for each population and across all populations; Observed (Ho) and expected (He) heterozygosities; * Significant deviation from Hardy-Weinberg equilibrium; +, Amplification within the expected range; -, No amplification; # , Polymorphic.

3.2.4 Acknowledgments

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3.3 Multilocus microsatellite analysis reveals high spatial genetic structure of *Phyllomedusa burmeisteri*, an endemic leaf-frog of the Brazilian Atlantic Forest [4]

3.3.1 Abstract

The Neotropical leaf frog, *Phyllomedusa burmeisteri*, is an endemic species with a widespread distribution throughout the Brazilian Atlantic Forest. Previous genetic studies revealed high levels of genetic substructure, including the presence of two highly divergent (p-uncorrected=7%) and well-supported mitochondrial lineages. Here we used 11 highly variable microsatellite loci from over 103 samples collected across the full range of *P. burmeisteri* to test whether the microsatellite data are congruent with or conflict with previously inferred phylogeographic patterns. We further investigate the relative roles of historical and contemporary processes in determining the current phylogeographic patterns. All population structure methods (Bayesian clustering and DAPC) consistently revealed four genetically distinct groups of populations. However, the pattern of population substructure was broadly discordant with that revealed by previous mtDNA analysis, with exception of the group uncovered for the Rio de Janeiro state. This coincidence across markers in revealing an evolutionary unit of *P. burmeisteri* in the region of Rio de Janeiro constitute an additional support for the recognition the role of this topographically complex area as a Pleistocene microrefugia. Taken together, our results suggest that the broad-scale patterns of spatial genetic structure of *P. burmeisteri* is still reflecting the combined effects of ancient vicariance events and current gene flow.

3.3.2 Introduction

The Neotropical leaf frog, *Phyllomedusa burmeisteri*, is an endemic species with a widespread distribution throughout the Brazilian Atlantic Forest (BAF), ranging more than 1000 km straight line between states of São Paulo and Bahia (Fig. 3.7). Within its distribution range it occurs on vegetation near standing waterbodies mainly in areas of dense ombrophylous and semi-deciduous forest, but also in areas of moderate anthropogenic disturbance (Bastos *et al*. 2010). The BAF is a well-known hotspot of amphibian biodiversity harboring 183 endemic species (SBH, 2013).

4 This work is in preparation for publication: Brunes TO, Alexandrino J, Haddad CFB & Sequeira F.

However, the high rates of deforestation has become a major concern, since it has represented a significant quantitative and qualitative habitat loss, and especially fragmentation of forests (Myers *et al*. 2000; Becker *et al*. 2007). Despite of the recent increased numbers of phylogeographic studies in amphibians of the BAF, comprehensive studies on factors affecting population structure and spatial genetic variation among and within populations are still scarce.

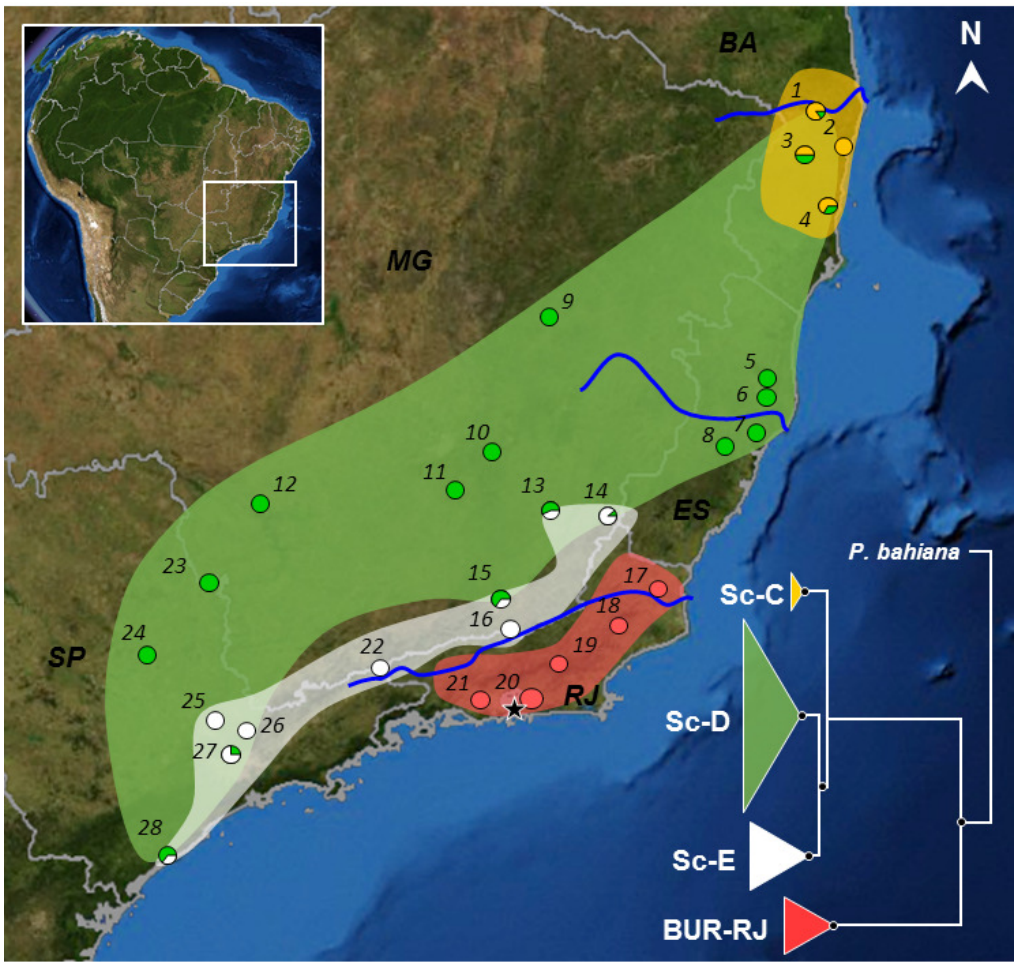

Fig. 3.7 Map of the study area along with genetic sampling locations (color of the dots correspond to mitochondrial-based defined haplgroups see below). Mitochondrial phylogeny for *Phyllomedusa burmeisteri* based on Bayesian analysis (ND2). Four haplogroups (Sc-C, D, E, and BUR-RJ) are defined and their approximate distributions across the Brazilian Atlantic forest are shown in corresponding colours on the map (Brunes *et al*. submitted). Blue lines indicates the major rivers of the Brazilian East Atlantic Basin; Jequitinhona, Doce, and Paraíba do Sul, respectively. Star indicate type locality. Black circles at nodes represent Bayesian posterior probabilities of ≥ 0.95. Brazilian states in italic: BA, Bahia; MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo.

In a recent phylogeographic study, Brunes *et al*. (in press; see Section 3.1) detected high levels of genetic substructure within P. burmeisteri, showing the presence of two highly divergent (p-uncorrected=7%; split time estimates ~1.3 Myr) and well-supported mitochondrial monophyletic clades. One of these clades is restricted to a small area coincident with the Serra do Mar in the Rio de Janeiro state,

being roughly delimited by the Paraíba do Sul river, while the other occurs in the extant distribution range of the species. Within this last clade, the same authors reported high levels of genetic substructuring, as evidenced by the detection of three well-supported and geographically delimited mtDNA subclades, whose divergence ranged between 1.5 to 1.9%; (*p*-uncorrected) (Fig. 3.7). This high genetic structure revealed at mtDNA level was not, however, recovered by the analysis of three nuclear gene genealogies, even when all were jointly analysed through the application of Bayesian model-based assignment methods. Notwithstanding, a coalescent-based multilocus species tree and the Bayesian species delimitation method (BPP) supported the two divergent mtDNA clades as distinct units with an independent evolutionary trajectory (Brunes *et al*. in press; see Section 3.1).

Genetic structure and differentiation within and among populations result from the interplay of both historic and contemporary evolutionary processes acting at various temporal and spatial scales. Accordingly, it is expected that patterns of population structure may differ among loci depending, among others, on the type of mutation process generating variation, mode of inheritance, and presence or lack of recombination (e.g. Zink & Barrowclough 2008). Indeed, there are an extensive list of studies documenting discordances between mtDNA and single copy nuclear genealogies, reporting, in general, a less resolution power of nuclear markers in inferring relatively recent evolutionary processes due to its higher effective population sizes and lower mutation rates when compared to mtDNA, that result in coalescent processes acting slower to produce a complete lineage sorting (e.g. Edwards & Beerli 2000; Broughton & Harrison 2003; Machado & Hey 2003; Zink & Barrowclough 2008).

Compared to single copy nuclear gene genealogies and mitochondrial markers, microsatellites evolve rapidly and has proven to be useful in revealing fine-scale population structure and/or inferring evolutionary processes on smaller temporal scales (e.g Sequeira *et al*. 2008). Accordingly, here we used information from 11 highly variable microsatellite loci, already developed for *P. burmeisteri* (Brunes *et al*. 2013), to provide insights into the relative roles of historical (e.g. river dynamics, geological activity or past climatic changes) and contemporary processes in determining the present phylogeographic patterns of *P. burmeisteri*. In particular, we focus in test major predictions based on previous study, addressing whether the microsatellite data are congruent with or conflict with the geographic boundaries of

the two highly divergent evolutionary units, and discuss the possible causes driven discordance in the geographic patterns of genetic variation among nuclear and mitochondrial markers.

3.3.3 Material and methods

Sample collection

We collected individuals of *Phyllomedusa burmeisteri* from all over the species range based on Brunes *et al. in press* (Section 3.1). To analyse the geographic distribution of the genetic variation of *P. burmeisteri* we used DNA information of 103 georeferenced individuals from 28 localities (Table 3.5 and Fig. 3.7). Tissue samples were obtained by field collecting trips from November of 2010/December of 2011 and by several tissues donations from different Brazilians herpetology's collections. Samples consisted of liver, toe clips, or muscle preserved in 100% ethanol. All collected frogs were fixed in 10% formalin solution for a week, preserved in 70% ethyl alcohol and deposited in the Célio F.B. Haddad amphibian collection at Departamento de Zoologia, Universidade Estadual Paulista "Júlio de Mesquita Filho" (CFBH), and in the herpetological collection at Departamento de Zoologia, Universidade Federal de Juiz de Fora (vouchers from Juiz de Fora locality). Tissue donations came from Vertebrate and Tissue Collection of Zoology Department of Universidade de São Paulo (USP), Herpetological Collection of Universidade Federal de Minas Gerais (UFMG), Herpetological Collection of Museu Nacional da Universidade Federal do Rio de Janeiro (MNRJ).

Markers and laboratory procedures

We used eleven microsatellite loci described by Brunes *et al*. (2013). Whole genomic DNA extraction from tissue samples followed procedures described in Sambrook *et al*. (1989). PCR amplifications were done following the conditions described in Brunes *et al*. (2013). Microsatellite genotyping was accomplished with fluorescent-labelled primers and an automated sequencer (ABI 3100, Perkin Elmer) using an internal size standard (Rox – genescan 350, Applied Biosystems). Analysis was done using GENESCAN software (Applied Biosystems), as described by Brunes *et al*. (2013).

Table 3.5 Genetic variability of microsatellite loci for population/locality (N≥5) of *Phyllomedusa burmeisteri*. Number of samples (N), allelic richness (Ar), observed (Ho) and expected heterozygosity (He), and inbreeding index (Fis). SL, sampling localities. ST, State abbreviations: BA, Bahia; MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo. *P < 0.05.

SL	Locality	ST	Long.	Latit.	N	He	Ho	A_R	F_{IS}	Cluster
1	Itapebi	BA	-39.5041	-16.0045	4	-	-	-	-	A
2	Porto Seguro	BA	-39.1805	-16.4081	2	-	-	-	-	A,B
3	Itabela	BA	-39.6781	-16.5653	2	-	-	-	-	A
4	Prado	BA	-39.3976	-17.1606	6	0.90	0.71	4.6	0.226*	A
5	Sooretama	ES	-40.0978	-19.1969	1	-	-	-	-	A
6	Linhares	ES	-40.0722	-19.3911	5	0.89	0.78	4.6	0.141	A
7	Aracruz	ES	-40.2733	-19.8203	8	0.84	0.67	4.2	0.212*	B
8	Santa Teresa	ES	-40.6051	-19.9525	2	-	-	-	-	A,D
9	S. João Evangelista	MG	-42.7494	-18.5461	1	-	-	-	-	A
10	Catas Altas	MG	-43.4378	-20.0562	4	-	-	-	-	A
11	Congonhas	MG	-43.8759	-20.4883	1	-	-	-	-	A
12	Furnas	MG	-46.2459	-20.6564	1	-	-	-	-	A,D
13	Viçosa	MG	-42.7515	-20.7523	7	0.85	0.74	4.2	0.141	A
14	Carangola	MG	-42.0836	-20.7986	12	0.88	0.72	4.6	0.194*	A,B,C
15	Juiz de Fora	MG	-43.3696	-21.7325	5	0.85	0.91	4.2	-0.076	A
16	Três Rios	RJ	-43.2159	-22.1124	1	-	-	-	-	A,C
17	C. dos Goytacazes	RJ	-41.4906	-21.5276	5	0.72	0.61	3.5	0.181	C
18	Sta. M. Madalena	RJ	-41.9384	-21.8811	4	-	-	-	-	A,C
19	Cach. de Macacu	RJ	-42.7491	-22.4257	7	0.80	0.61	3.7	0.249*	A,C
20	Niterói	RJ	-43.1045	-22.8798	1	-	-	-	-	A
21	Rio de Janeiro	RJ	-43.5315	-22.8278	1	-	-	-	-	C,B
22	S. José Rio Pardo	SP	-46.8727	-21.5493	1	0.70	0.62	3.3	0.126	A
23	Rio Claro	SP	-47.6755	-22.3472	5	-	-	-	-	D
24	Queluz	SP	-44.7739	-22.5369	1	-	-	-	-	D
25	Jundiaí	SP	-46.8172	-23.1647	8	0.80	0.60	3.8	0.253*	D
26	Nazaré Paulista	SP	-46.4191	-23.2373	3	-	-	-	-	D
27	“Grande” São Paulo	SP	-46.6361	-23.5475	2	-	-	-	-	D
28	Iguape	SP	-47.5414	-24.6981	3	-	-	-	-	D

Data analysis

Measures of genetic diversity based on microsatellites, including mean number of alleles (A), allelic richness (AR), and observed (H0) and expected (HE) heterozygosity (Nei 1978) and the inbreeding coefficient (FIS) were calculated using GENETIX v. 4.01 (Belkhir et al 2000). To test for linkage disequilibrium (LD) and departures from Hardy-Weinberg equilibrium (HWE) among the loci and samples, we used GENEPOP 3.3 (Raymond & Rousset 1995). All probability tests were based on MCMC simulations (Guo & Thompson 1992; Raymond & Rousset 1995) using default values, with significance levels adjusted for multiple tests using sequential Bonferroni corrections ($\alpha = 0.05$; Rice 1989).

Estimates of population genetic differentiation were determined by calculating pairwise values of FST (Weir & Cockerham 1984) in GENETIX, Fisher's exact test, implemented in GENEPOP, and Nei's modified chord distance DA (Nei *et al.* 1983) with the software POPULATIONS v. 1.2.28 (http://www.pge.cnrs-gif.fr/bioinfo/populations/index.php). Statistical significance of FST values among populations was tested using 1000 randomisations, adjusting the critical probability level with the sequential Bonferroni correction. To test for population substructure, the FST values with 95% bootstrapped confidence intervals (CI) were used.

Population structure

Bayesian model-based clustering

Population structure and genetic admixture analysis was investigated with no *a-priori* assignment of individuals to populations using two Bayesian model-based clustering programs: i) STRUCTURE v. 2.3.3 (Pritchard *et al*. 2000) that implements a non-spatial Bayesian MCMC clustering method; and ii) TESS v. 2.3 that incorporates a spatially explicit clustering method (Chen *et al*. 2007), using a Hidden Markov Random Field (HMRF) method to model spatial dependency (ψ), which measure the intensity of two neighbour individuals belonging to the same genetic cluster. In STRUCTURE, we first performed, under the admixture ancestry model, ten independent runs for each K ranging from 1 to 12, using 5x105 MCMC repetitions and discarding the first 5x104 iterations as burn-in. We estimated the smallest number of K (clusters) that captures the most population structure in the data monitoring the estimated log posterior probability of the data (ln Pr (X/K) (Pritchard *et al*. 2000), estimating the second-order rate of change of the likelihood function (ΔK,b) (Evanno *et al*. 2005), and examining the mean confidence assignment of all individuals (q) to their most probable clusters. To help in selection of the most optimal value for K, post processing STRUCTURE runs were done by the software STRUCTURE HARVESTER v0.3 (Earl & VonHoldt 2012).

For analysis in TESS, because we have only specific sites sample spatial coordinates, which could interfere with the accuracy of the individual assignment process (Durand *et al*. 2009), we first use the "Generate Spatial coordinates" function to display random individual coordinates within each site sample. For that, each latitude and longitude was permuted slightly by a standard deviation of 0.1. Then, we

performed several pilot runs for K from 2 to 12 to test for different spatial dependency (ψ) values (0.3-0.9) using a conditional autoregressive admixture model (CAR) and linear trend surface. These runs were performed with a burn-in of 20 000 sweeps and a run-length of 100 000 iterations. Since varying spatial dependency values (ψ) not changes the overall assignment probabilities, we performed 15 runs for a number of clusters ranging from K= 2 to 12, using ψ = 0.7, maintaining the same run settings described above. The best K was determined plotting DIC (Deviation information Criterion) against K and choosing the values of K that correspond to a plateau of the curve (Durand *et al*. 2009). For the most probable K, CLUMPP v.1.1.1 (Jakobsson & Rosenberg 2007) was used to label switching of clusters across replicates and to estimate average matrices of ancestry membership proportion (admixture Q matrix output) over the several replicate simulations performed in both STRUCTURE and TESS. Estimated average admixture Q matrices were graphically represented using the DISTRUCT software (Rosenberg *et al.* 2004). Results obtained with both TESS and STRUCTURE was spatially interpolated applying the KRIGING interpolator using the program R v.2.12.0. Geographical distribution of the four genetic groups (A–D) was displayed through the spatial interpolation method based on distance weighted interpolation after a threshold classification of the original model (0.7), built according to Structure membership coefficients (defined hereafter as core areas).

Discriminant Analysis of Principal Components (DAPC)

The genetic variability of microsatellites was explored by means of a DAPC analysis (Jombart *et al*. 2010), which does not rely on a particular population genetics model, being therefore free of assumptions about Hardy-Weinberg equilibrium or linkage disequilibrium. Preliminarily, data were grouped using k-means, a clustering algorithm which finds a given number of clusters maximizing the variation between groups. The optimal number of clusters was identified by running k-means with increasing values of k=28). Clustering solutions for different k values were compared calculating Bayesian Information Criterion (BIC). The 'best' solution corresponded to the lowest BIC (Jombart *et al.* 2010).

3.3.4 Results

Genotypic data

For the 11 microsatellite markers examined in this study, the total number of alleles ranged from 16 to 33 with a mean of 21 per locus, while allelic richness ranged between 9.7 and 13.6 (11.4 on average) (Table 3.6). Measures of genetic diversity did not vary considerably across populations/localities and clusters as inferred by STRUCTURE analysis (Table 3.5). The average observed heterozygosity was lower than the expected in several populations and in all STRUCTURE clusters, which indicate the presence of heterozygosis deficit (Table 3.5 and 3.6). There were no pairs of loci with significant linkage disequilibrium after Bonferroni correction. HWE tests representing every polymorphic locus/population combination (after Bonferroni correction) showed significant deviation in four tests (in a total of 110) of three loci (Phybu 17, 21 and 27), while for STRUCTURE clusters deviations were detected in 13 tests (in a total of 44) for seven loci (Phybu 6, 4, 11, 17, 21, 26 and 28). In both population and STRUCTURE clusters analysis, there were only minor differences in allelic diversity (N), observed heterozygosity and expected heterozygosity (Ho and He) and inbreeding index (FIS) (Table 3.5).

Table 3.6 Genetic variability of 11 microsatellite loci for each cluster identified in *Phyllomedusa burmeisteri* using STRUCTURE. Number of samples (N), average number of alleles (A), allelic richness (A_R), observed (H_O) and expected heterozygosity (H_E), and inbreeding index (F_{IS}). Mean confidence assignment of all individuals to their most probable clusters and percentage (in parenthesis) of individuals assigned to one of four clusters with a probability > 0.80 (*q*). *$P < 0.05$.

Cluster	N	A	A_R	H_O	H_E	F_{IS}	*q*
A	57	21.1	11.6	0.76	0.91	0.179*	0.92 (72.2)
B	11	8.7	8.2	0.57	0.68	0.243*	0.88 (82.9)
C	12	9.0	8.7	0.65	0.80	0.246*	0.93 (58.3)
D	23	10.3	8.0	0.67	0.81	0.203*	0.91 (86.6)

Genetic differentiation and population structure

We used pairwise FST estimates and genetic distance DA (Nei *et al*. 1983) to examine genetic differences between the identified four clusters and the 10 populations/localities (Table B.2). With exception of population 4 and 6, pairwise measures of differentiation revealed moderate to high levels of population

divergence, being significantly different in all pairwise comparisons, including between the four clusters identified by STRUCTURE (Table 3.7). The highest among cluster pairwise FST and Da estimates were found between the clusters B and C (FST=0.165; DA=0.552) and the lowest pairwise comparison was between the clusters A and D (FST=0.050; DA=0.318). For population-based analysis, the highest among cluster pairwise FST and DA estimates were found between populations 17 and 22, (FST=0.246; DA=0.800), while the lowest pairwise comparison was between the populations 4 and 6 in the case of FST, and between 22 and 25 for DA (FST=0.019;DA=0.501, respectively). Highly significant genetic differentiation among clusters was also found using Fisher's exact test ($p < 0.001$).

Table 3.7 Comparisons of pairwise F_{ST} values above the diagonal and pairwise values of Da Nei et al's 1983 genetic distance for genetic clusters A-D in the *Phyllomedusa burmeisteri*. In comparisons of pairwise F_{ST} *P < 0.05.

Cluster	A	B	C	D
A	-	0.098*	0.056*	0.050*
B	0.383	-	0.165*	0.153*
C	0.390	0.552	-	0.103*
D	0.318	0.531	0.538	-

The Bayesian structure analysis did not reveal an unequivocal number of genetic clusters. First, ΔK statistic peaked at K=2, suggesting that the uppermost hierarchical structure for the data set consists of 2 groups, but it was clear from the analyses that biologically meaningful clusters were observed at higher values of K. Indeed, Ln Pr (X/K) increased from K=2 to K=6, but an increasing of K above 4 did not represent a significant increase of Ln Pr (X/K) and assignment uncertainties were apparent as reflected by a large standard deviation of the mean value after K=4 (Fig. B.3). Furthermore, the analysis of the patterns of cluster composition examining the mean confidence assignment of all individuals (q) to their most probable clusters (an indicative measure of the assignment robustness), showed that for K>4, q <0.80 (data not shown). Accordingly, we accepted K=4 clusters as the value that captures the most population substructure in the data (Fig. 3.8). In agreement, results performed by TESS based on the inflexion point for the mean DIC value found K=4 as the most likely number of clusters that fits the data. In fact, the DIC curve decreases sharply until K=4, and increasing K above this value did not represent a

significant decrease of DICs (Fig. B.4). At this K value (K=4), this Bayesian clustering analysis, using spatial information, recovered very similar clusters to those of Structure (Fig. 3.8c and Fig. B.5).

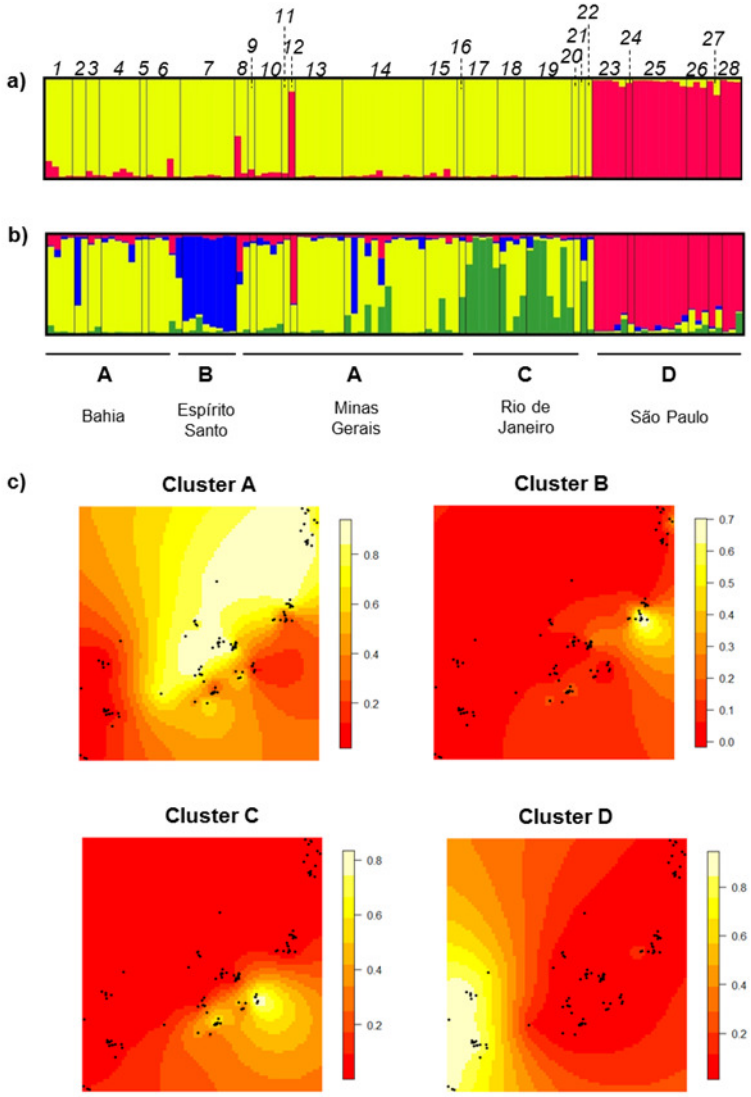

Fig. 3.8 Bayesian admixture analysis of *Phyllomedusa burmeisteri* based on 11 microsatellite loci using Structure for K=2 (a) and K=4 (b). Each vertical bar represents the membership coefficient (q) for each individual. Numbers above bars indicate sampling localiteis (Table 3.5 and Fig. 3.7), and A - D, below, designate four genetic clusters and geographic location. Spatially explicit estimate of population structure based on the STRUCTURE results for K=4 (c) Results were obtained across 5 runs using CLUMPP (Jakobsen & Rosenberg 2007) and displayed through the spatial interpolation method (Kriging). Each of K=4 clusters are displayed in a separate graph, in which the highest posterior probabilities are in white and the lowest are in red (see details in material and methods). Black dots represent each individual.

Remarkably, the 2 groups identified by ΔK statistic separates populations/localities from S. Paulo state and the remaining populations (Fig. 3.8a), while increasing K to 4 consistently revealed three additional clusters out of S. Paulo (cluster D): 1) Cluster B, representative of a small area of Espírito Santo; 2) Cluster C, restricted to Rio de Janeiro; and, 3) cluster A, the largest one, that includes populations from Bahia to the interior part of the Atlantic Forest corresponding to Minas Gerais (Fig. 3.8b). At this level of population substructure, a majority of

individuals (N=79) was assigned to one of four clusters with q>0.80 (Fig. 3.8b). For 21 individuals, STRUCTURE assigned their genome to two clusters, which could be evidence of recent admixture between two subpopulations, while 3 individuals are not assigned strongly to any cluster (Table 3.5 and Fig. 3.8b). The Figure 3.9 shows the geographical distribution of the four genetic groups (A-D), built according to Structure membership coefficients, and revealed that the core areas A and D are significantly area than B and C.

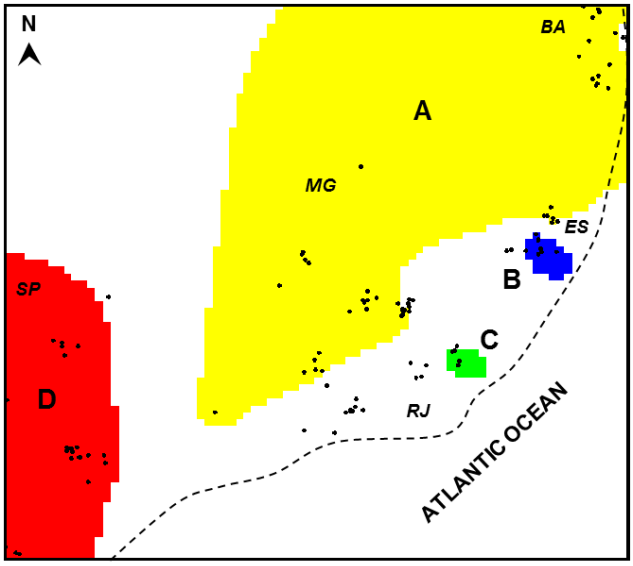

Fig. 3.9 Geographical distribution of the core areas of the four genetic groups (A–D) based on distance weighted interpolation of *Phyllomedusa burmeisteri* individuals. Core areas are defined after a threshold classification of the original model (0.7), built according to Structure membership coefficients for *K*=4 (c). Dashed line represents the limit of the coast. Brazilian states: BA, Bahia; MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo.

In DAPC analysis, we retained 23 principal components of PCA, which accounted for approximately 60% of the total genetic variability. Following the Bayesian information criterion (BIC), the subdivision inferred by DAPC (Fig. 3.10) was strikingly similar to the four clusters identified by Bayesian clustering analysis (Fig. 3.8b). DAPC analysis showed a hierarchical structure, where three groups of genetically closer clusters can be identified ({1}, {4}, and {2, 3}).The first principal component clearly differentiates individuals from the Rio de Janeiro cluster C (green) from the remaining clusters. The second principal component separates clusters 4 and 2 (S. Paulo) from the two closely related clusters 1 (Bahia and Minas Gerais) and 3 (Espírito Santo).

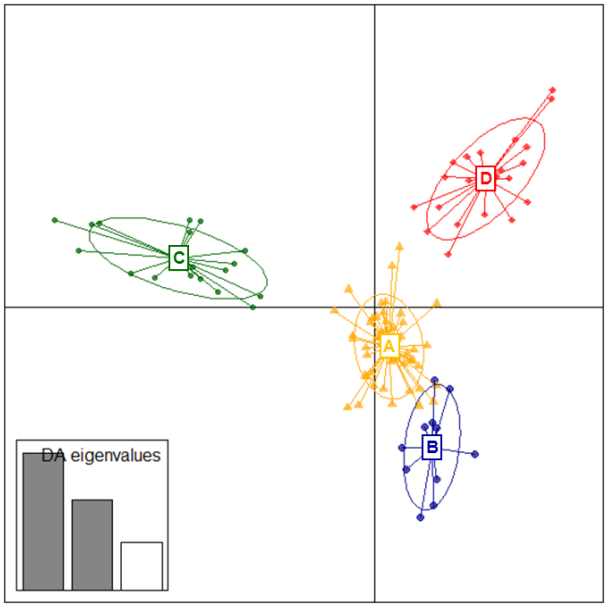

Fig. 3.10 Plot of the first two axes obtained in the Discriminant Analysis of Principal Components (DAPC) using 11 microsatellite data for *Phyllomedusa burmeisteri* individuals. Color circles, triangles and diamonds represents each individuals. Color codes identify the same four genetic clusters obtained with STRUCTURE (Fig. 3.8).

3.3.5 Discussion

In this study, we used 11 microsatellite loci to further assess whether patterns of population genetic structure are concordant with the spatial structuring of mtDNA variation of *P. burmeisteri* across its entire distribution range in the Brazilian Atlantic Forest. All methods (Bayesian clustering and DAPC) consistently revealed the partition of genetic variation in four clusters (K=4). The first, includes populations from the southern part of Bahia to the interior part of the Atlantic Forest, encompassing most of the eastern region of the Minas Gerais state (cluster A); the second, occurs in a small area of Espírito Santo close to Doce river (cluster B); the third, is restricted to Rio de Janeiro state (cluster C); and, the fourth, ranging from the coast to the interior part of the S. Paulo state (Fig. 3.8). This pattern of population substructure is broadly discordant with that revealed by the mtDNA analysis (Brunes *et al*. in press; see Section 3.1), according to which most populations from the states of S. Paulo, Minas Gerais, Espírito Santo and Bahia are clustered in the same group. The only exception was the cluster C, which is nested within the highly divergent mtDNA clade (BUR-RJ).

Discordance between the biogeographic patterns obtained from mitochondrial DNA and those observed in the nuclear genome is not unexpected given differences in inheritance mode and ploidy of these types of markers. Indeed, discordant patterns have been referred for a wide range of organisms (e.g. Toews & Brelsford 2012; Mattioni *et al*. 2013), for which introgression, stochastic variation, demographic effects and sex-biased asymmetries are

among the most evoked explanations. In particular, several studies have demonstrated a tendency of microsatellites to mask signals of historical diversification of highly divergent mtDNA lineages, showing comparatively less levels of differentiation (Neumann *et al*. 2005; Sequeira *et al*. 2008). Despite of both mtDNA and microsatellites are concordant in revealing one genetically distinct group of *P. burmeisteri* in Rio de Janeiro state, this concordance is not paralleled by levels of genetic differentiation. Whereas the divergence between BUR-RJ and the extant clades is approximately six fold higher, for microsatellites the divergence as measured by FST and Da genetic distance is roughly similar across clusters. Given the typical fast mutation rates of microsatellites, it is possible that allele size homoplasy, through a combination of mutation constraints and back mutations, and/or contemporary gene flow may account for the lack of pronounced levels of differentiation showed by microsatellites. Following our results from Bayesian clustering Structure analysis (Figs. 3.8b-c, Fig. 3.9), some localities from RJ belonging to mtDNA BUR-RJ clade either comprise individuals assigned to two clusters (C and A) or exclusively assigned to cluster A, which could be an evidence of recent gene flow. Indeed, with exception of population 17 that corresponds to the core area from the RJ cluster C, populations 18 and 19 exhibits high levels of admixture, and the individual from SL 20 is assigned to Cluster A (Fig. 3.9).

Despite the evidence we observed for both different levels of differentiation and geographic range across markers for the Rio de Janeiro group, what factors could be evoked to explain the origin and persistence of this regionally differentiated group? It is well-known that spatial variation in diversity results from the interplay of ecological and evolutionary forces acting over historical and contemporary time scales (Fritz *et al*. 2013; Graham *et al*. 2014). This group occurs in a topographically and phytogeographically complex region that encompasses the valley of Paraíba do Sul and several mountain ranges belonging to Serra do Mar complex, in particular Serra do Desengano, Três Picos and Serra dos Órgãos, which are characterized by steep altitudinal (>1000 m), climatic and phytophysiognomy transitions (IBGE 2012). These characteristics together with palynological evidences of past landscape complex changes due to Quaternary climatic oscillations likely have created conditions for diversification and persistence of populations. Indeed, the occurrence of high levels of endemic species (Rocha *et al.* 2004) and/or several evolutionary units (Pellegrino *et al*. 2005; Clemente-Carvalho *et al*. 2011; Fusinatto *et al*. 2013; Gehara *et al*. 2013; Amaral *et al*. 2013) constitute strong evidences favoring the role of this region as a Pleistocene microrefugia. Taken together, our results suggest that the broad-scale patterns of spatial genetic structure are largely derived by the combined effects of a historical isolation, as evidenced by Brunes *et al*.

(in press, Section 3.1) based on mtDNA analysis, and contemporary gene flow, as suggested in this study. Indeed, the mtDNA usually tends to higher stationarity due to lower effective population size, retaining thus signatures of historical processes while microsatellites are more likely to form waves of introgression due to higher effective population sizes.

Other striking result of this study corresponds to the observation of a well-differentiated cluster exclusively at nuclear level in a small area of Espírito Santo state located south of Rio Doce. One possible explanation for the origin of this cluster is associated with its geographic location in between of two main rivers of Brazilian Atlantic Forest (Doce and Paraiba do Sul rivers) that likely act as barriers to gene flow. Indeed, individuals collected in the northern margin of river Doce (SL 6) were assigned to cluster A, while individuals from south of Paraiba do Sul river were assigned to cluster C. Interestingly, however, is that the results from mtDNA analysis employed by Brunes *et al.* (in press, Section 3.1) showed the co-occurrence of mtDNA haplotypes of *P. burmeisteri* and the closely related *P. bahiana* in this same area (SL 7). Four individuals bearing two *P. bahiana* found in Espírito Santo state were assigned to *P. burmeisteri* on the basis of scnDNA, suggesting that the presence of both haplotypes in this region may be a remnant of a wider distribution of *P. bahiana* in the past (Brunes *et al*. in press, Section 3.1). Accordingly, it is possible that the unique genetic composition of this cluster b may reflect an older hybridization event in the Espírito Santo region between *P. bahiana* and *P. burmeisteri*, and could be erroneously interpreted as population structure driven by genetic isolation.

Finally, it is important to highlight that the presence of two wide genetically uniform areas corresponding to clades A and D should be viewed with caution due to the small sample sizes from regarding the total range. In fact, more than half of our sample set is concentrated near the coast, and therefore it is possible that small sample sizes may result in insufficient power of the methods here employed to detect additional levels of structuring. Accordingly, additional samples from both of those areas are needed in order to better evaluate the spatial variation of population genetic structure.

3.3.6 Acknowledgements

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Capítulo 4

Inferência de padrões filogeográficos combinando teste de hipótese e modelos paleoclimáticos

4.1 Ancient divergence and recent population expansion in a leaf frog endemic to the southern Brazilian Atlantic forest[5]

4.1.1 Abstract

Aim The evolutionary history of Neotropical organisms has been often interpreted through broad scale generalizations. One of the most accepted models of diversification for the Brazilian Atlantic forest (BAF) rely on putative historical stability of northern areas, and massive past habitat replacement of its southern range. Here, we use the leaf frog *Phyllomedusa distincta*, endemic to the southern BAF, to better understand diversification patterns within this underexplored rainforest region.

Location Southern Brazilian Atlantic forest

Methods We used an integrative approach coupling fine scale sampling and multilocus sequence data, with traditional and statistical phylogeographic (multilocus Approximate Bayesian Computation) methods in exploring alternative hypotheses of diversification. We also employed species paleodistribution modelling to independently verify habitat stability upon a spatially explicit model.

Results Our data support two divergent lineages with coherent geographic distribution that span throughout northern and southern ranges. Demographic estimates suggested the Southern lineage has experienced a recent population expansion, whereas the Northern lineage remained more stable. Hypothesis testing supports a scenario of ancient vicariance (mid-Pleistocene) with recent population expansion. The paleodistribution model revealed habitat discontinuity during the Last Glacial Maximum (LGM) with one area of putative stability within the range of the Northern lineage.

Main conclusions Evidence on genetic structure, demography and paleodistribution of Phyllomedusa distincta support a historically heterogeneous landscape for the southern BAF, with both areas of forest stability and regions where forest occupation is probably recent. We also associate the southern end of the Cubatão shear zone to a phylogeographic break for first time in the BAF. Taken together, our results argue

5 This study is presently submitted to an international journal in the science-citation index: Brunes TO, Thomé MTC, Alexandrino J, Haddad CFB & Sequeira F.

for the idea of multiple mechanisms originating diversity in this biome and underscore the need of fine scale data in revealing more detailed pictures.

4.1.2 Introduction

To describe and understand patterns of biological diversification in the Neotropics has been a challenge for many evolutionary biologists, with the focus being on the tempo and mode of processes underlying its large biodiversity (Rull, 2008). Encompassing enormous habitat heterogeneity, high numbers of endemic species and a frightening level of deforestation (Myers *et al*., 2000; Ribeiro *et al*., 2009), the Brazilian Atlantic forest (BAF) has been the focus of most recent studies in diversification of South American taxa (Turquetto-Zolet *et al.* 2013). Advances at genetic and spatial analyses fomented a substantial increase in the knowledge regarding species diversification, especially by combining statistical phylogeography and species distribution modelling (SDM; reviewed in Alvarado-Serrano & Knowles 2013). In the BAF, such tools contributed by both originating a spatially explicit hypothesis of habitat persistence (Carnaval & Moritz 2008) and by providing the means to test it (Carnaval *et al*. 2009). This resulted in a model where organisms found refuge in the north and central areas of the BAF during the Last Glacial Maximum (LGM), and subsequently colonized the southern region favored by Holocene rainforest recovery (Carnaval & Moritz, 2008; Carnaval *et al*., 2009). Nonetheless, a set of studied taxa show patterns that support southern habitat persistence, highlighting instead the role of rivers and neotectonic activity during the Late-Tertiary and the Quaternary as main promoters of diversification (Pellegrino *et al*., 2005; Grazziotin *et al*., 2006; Thomé *et al*., 2010; Amaral *et al*., 2013).

Organisms widespread in the BAF have been frequently considered as best models to uncover the evolutionary history of the region. However, a recent ordination-based analysis of climatic data identified the northern and southern BAF as two distinct climatic regions (Carnaval *et al*., 2014), raising the question that studies with taxa of more restricted distributions may be more appropriate to unveil the main diversification patterns of each region. Furthermore, whereas studies on widespread taxa frequently suffer from coarse sampling, extensive revisions warn for the need of fine-scale data to avoid broad generalizations over more complex histories (Martins, 2011; Silva *et al*., 2012). Here, we use the leaf frog *Phyllomedusa*

distincta to better understand diversification patterns of species endemic to southern BAF. *Phyllomedusa distincta* is the only of five species in the group of *Phyllomedusa burmeisteri* with a range restricted to ombrophilous dense forest. A previous study found two highly supported mitochondrial clades showing contrasting levels of genetic variability. Insufficient sampling prevented further investigation on the demographics causing this pattern, and authors tentatively associated this divergence to geomorphologic events in southern BAF (Brunes *et al*., 2010). Here, we drastically extended the geographic and genetic sampling of *P. distincta* and coupled traditional and statistical methods of phylogeographic analysis with species paleodistribution modelling methods to: i) verify if previously reported mtDNA structure is also presented in nuclear DNA; ii) estimate demographic parameters, construct and test hypotheses of contrasting demographic scenarios, and finally, iii) compare phylogeographic inference with independent spatially explicit hypothesis of habitat persistence obtained through species paleodistribution modeling.

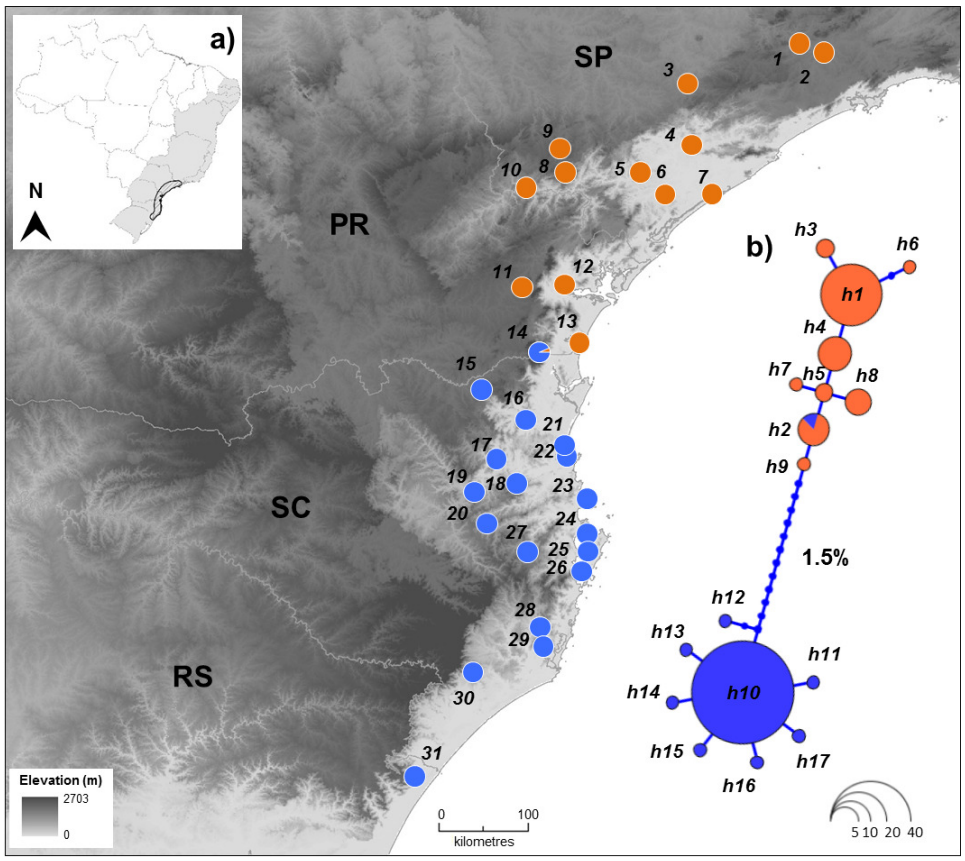

Fig. 4.1 Geographic distribution of *Phyllomedusa distincta* in the southeast Brazilian Atlantic Forest (a), and mitochondrial haplotype genealogy derived from ND2 sequences (b). Sampling localities and haplotype numbers are indicated (see Table 4.1). The circle area of each haplotype is proportional to its frequency. Mutations are edges. Percentages indicate uncorrected pairwise distance between haplogroups. Brazilian states: SP, São Paulo; PR, Paraná; SC, Santa Catarina; and RS, Rio Grande do Sul.

4.1.3 Materials and methods

Sampling and sequencing procedures

We obtained a total of 118 tissue samples of *P. distincta* from field collecting trips and tissue donations from Brazilian herpetological collections (see Acknowledgments). Samples consisted of liver, toe clips, or muscle preserved in 100% ethanol. To avoid misclassification due to the presence of hybrid individuals between *P. distincta* and *P. tetraploidea* that occur in Ribeirão Branco, south of São Paulo state (Haddad *et al*., 1994), we previously confirmed the ploidy number of all individuals from this locality by cytogenetic analysis (Gruber *et al.* 2013; Sanae Kasahara, unpublished). We sampled a total of 31 localities (Sampling localities, SL) with elevation ranging from sea level to ~ 915 meters altitude (Table 4.1 and Fig. 4.1a). We georeferenced all samples with GPS or used the coordinates of the closest town.

Table 4.1 Columns indicate sampling locality information, sample sizes, and mitochondrial haplotype numbers of *Phyllomedusa distincta*. State abbreviations (ST): SP, São Paulo; PR, Paraná; SC, Santa Catarina; RS, Rio Grande do Sul.

SL	Locality	ST	Longitude	Latitude	N	h (ND2)
1	Carapicuíba	SP	-46.8356	-23.5225	2	1
2	São Paulo	SP	-46.6279	-23.5752	1	1
3	Pilar do Sul	SP	-47.7164	-23.8131	1	2
4	Juquiá	SP	-47.6785	-24.328	8	1,2,3
5	Eldorado	SP	-48.0804	-24.5337	5	1,4
6	Pariquera-Açu	SP	-47.8811	-24.715	1	1
7	Iguape	SP	-47.5414	-24.6981	4	1,4
8	Iporanga	SP	-48.7003	-24.5328	3	1,4,5
9	Ribeirão Branco	SP	-48.7430	-24.3586	8	1,2,6
10	Adrianópolis	PR	-49.0315	-24.6404	6	1,4,5,7
11	Piraquara	PR	-49.0444	-25.4422	2	8
12	Antonina	PR	-48.7119	-25.4286	1	9
13	Guaratuba	PR	-48.5747	-25.8828	4	2,8
14	Garuva	SC	-48.8887	-25.9807	8	2,10
15	São Bento do Sul	SC	-49.3786	-26.2503	2	10
16	Guaramirim	SC	-49.0032	-26.5099	1	10
17	Timbó	SC	-49.2594	-26.8075	5	10
18	Blumenau	SC	-49.0911	-27.0289	3	10,11
19	Apiúna	SC	-49.4142	-27.098	2	10,12
20	Vidal Ramos	SC	-49.3304	-27.3604	1	10
21	Barra Velha	SC	-48.6918	-26.7242	4	10
22	Santa Lídia	SC	-48.6537	-26.833	8	10
23	Porto Belo	SC	-48.5168	-27.1238	6	10
24	Florianópolis “North”	SC	-48.5069	-27.4585	11	10,13
25	Florianópolis “Center”	SC	-48.5101	-27.5966	1	10
26	Florianópolis “South”	SC	-48.5438	-27.7307	1	14
27	Angelina	SC	-49.0049	-27.5885	3	10,15
28	São Martinho	SC	-48.8998	-28.188	1	10
29	Imbituba	SC	-48.8536	-28.3111	2	16,17
30	Treviso	SC	-49.4575	-28.5156	8	10
31	Dom Pedro de Alcântara	RS	-49.8928	-29.3806	5	10

Genetic markers included one fragment of the mitochondrial NADH dehydrogenase subunit 2 gene (ND2), and three nuclear fragments: i) a segment of exon 2 and intron 2 of the cellular myelocytomatosis (C-myc2), ii) a segment of exon 2 of chemokine receptor 4 (CXCR4), and iii) a segment of β-fibrinogen intron 7 (β-fibint7). We obtained whole genomic DNA through tissue samples digested in lysis buffer and Proteinase K and using QIA Quick DNEasy columns (Qiagen, Inc.) according to the manufacturer's protocol. We performed PCR amplification and sequencing of ND2 and β-fibint7, and of C-myc2 and CXCR4 following Brunes *et al.* (2010) and Brunes *et al*. (2014), respectively. We downloaded additional sequences from GenBank (Faivovich *et al*. 2010; Brunes *et al*. 2010; see Table C.1). We edited alignments and correct by eye in BIOEDIT 7.0.5.2 (Hall, 1999).

Polymorphisms, pairwise distance, and recombination

We calculated polymorphism values in DNAsp 5.10 (Librado & Rozas, 2009), for both the entire dataset and within main mitochondrial and nuclear groups including: number of segregation sites (S), number of haplotypes (h), haplotype diversity (Hd), number of singletons haplotypes (Sn), and the population mutation parameter theta, computed from the number of S (Θw; Watterson, 1975). We generated 95% confidence intervals with 10,000 coalescent simulations.

To resolve the allele phase of nuclear markers we used the software PHASE 2.1 (Stephens *et al*., 2001) with the assistance of SeqPHASE (Flot, 2010). We performed multiple independent runs for each gene with different seeds for the random-number generator and $1.0x10^6$ iterations with the default values. We checked haplotype estimation through analysis of consistency across runs. We selected the haplotype reconstructions with higher probabilities. Additionally, we identified multiple-base insertions or deletions (indels) in two nuclear loci (β-fibint7 and C-myc2) with the mutation detection tool in CodonCode Aligner, and then reduced indels to a single evolutionary step following Brunes *et al*. (2010). We further evaluated the presence of recombination events through the Difference of Sums of Squares (DSS) test implemented in TOPALi 2.5 (Milne *et al*., 2004) using all phased haplotypes, a window size of 70 bp, steps 10 bp-long, and 100 bootstrap repetitions. We used Alignment Transformation Environment, ALTER (Glez-Peña *et al*., 2010) to transform datasets to the input formats of down-the-line software used for analyses.

Phylogeographic and population structure

Following Salzburger *et al*. (2011) results, we used the haplotype genealogy method to clarify the patterns of distribution of *P. distincta*. This method avoids the presence of reticulation and provides a "clear" scenario to sets of highly similar haplotype sequences by turning phylogenetic trees into haplotype networks. Thus, we produced haplotype genealogies for both mitochondrial and nuclear fragments starting from a maximum parsimony (MP) tree though DNAPARS available in PHYLIP package 3.69 (Felsenstein, 2005). Then, we converted the best parsimony tree into a haplotype genealogy in a beta version of the Haploviewer (available at: http://www.cibiv.at/~greg/haploviewer).

We evaluated the nuclear multilocus structure using a combined distance matrix (POFAD; Joly and Bruneau 2006) and a Bayesian clustering method (STRUCTURE; Pritchard *et al.* 2000). In both cases, we used the sequence information for at least two nuDNA fragments per individual, in a total of 22 individuals from 18 localities (Table 4.1). We calculated *p-uncorrected* distances between individuals for each nuDNA fragment in MEGA 5.2.2 and combined into a multilocus distance matrix in POFAD. Both standardized and non-standardized matrices were produced in order to evaluate the possible understatement of attribution of the same weight for all matrices. We visualized the multilocus distance network in Splits Tree 4.12.3 (Huson & Bryant, 2006) via NeighborNet method. We inferred population clusters with STRUCTURE (Pritchard *et al*., 2000) via allele frequency converted through the program xmfa2struct (available at: http://www.xavierdidelot.xtreemhost.com/clonalframe.htm). We performed analysis under the admixture ancestry model, with five independent runs for each K ranging from 1 to 10. We discarded the first 1×10^5 MCMC iterations as burn-in and counted the next 25×10^4 repetitions. We found the best K value via the on-line program Structure Harvester v.0.6.93 (Earl & VonHoldt, 2012) by monitoring the estimated log posterior probability of the data (ln Pr (X/K)) (Pritchard *et al*., 2000), and estimating the second-order rate of change of the likelihood function (ΔK) (Evanno *et al*., 2005). Finally, we assembled the results of the five independent runs in the program CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007) and checked for biologically meaningful population clusters.

Divergence time, ancestral population parameters and gene flow estimates

We applied an isolation-with-migration model (IM; Wakeley & Hey 1997; Nielsen and Wakeley 2001; Hey & Nielsen 2007) to multilocus data as implemented in the program IMa2 (Hey, 2010) to explore the speciation history of *P. distincta*. We used the HKY model of evolution (Hasegawa *et al*., 1985) for both mitochondrial and nuclear DNA. We ran the program under Metropolis Coupled MCMC, using ten chains with linear heating mode. We conducted multiple preliminary runs to assess mixing of the chains, as well as to determine appropriate priors for the parameters. After this, we ran the program three times with different random seeds. For each simulation the length was > 10×10^6 steps, where the first 10×10^5 was discarded as burn-in. We checked convergence of the Markov chain simulations to stationary distribution by monitoring multiple independent runs using different random number seeds (similar posterior distributions for each parameter across independent runs) and by assessing effective sample sizes (ESSs) values (ESS > 100), trendline plots, and swapping rates between chains over the course of the run. We adopted a strict molecular clock and uniform priors on substitution rates of 0.957% substitutions/site/year for ND2, estimated by (Crawford, 2003) for Leptodactylidae species and previously used to estimate splits time in *Phyllomedusa burmeisteri* species group (Brunes *et al*., 2010). For the nuclear genes we used the mutation rate scalars estimated by the program. We converted divergence time, current and ancestral population sizes (θ), and population migration rates (2NM) to demographic units following author's instructions. We assumed a generation time of 1 year.

Demographic history

To evaluate if mitochondrial and nuclear groups present deviations from the neutral theory we computed Tajima's D (Tajima, 1989), Fu's Fs (Fu, 1997) and R^2 test (Ramos-Onsins & Rozas, 2002) with significance of values checked through 10,000 coalescent simulation in DNAsp 5.10.

We used the extended Bayesian skyline plot (EBSP) to reconstruct the historical demography over time of each lineage. This coalescent method is relatively sensitive to estimate the population size history from multiple unlinked loci, even with small number of individuals (Heled & Drummond, 2008). We believe that the use of a multilocus skyline approach is paramount to reduce the coalescent error and

increase the ability to detect population bottlenecks (see revision in Ho & Shapiro 2011). We conducted analysis in BEAST 1.7.4 (Drummond *et al*., 2012) with the whole molecular dataset (one mtDNA and tree nuDNA fragments) using the linear demographic function. We selected models of nucleotide evolution in jModelTest v.0.1.1 (Posada, 2008) under the Akaike information criterion (AIC; Akaike 1974). Then, we applied TN93 to ND2 and C-myc2, and HKY model to CXCR4 and β-fibint7. We selected a strict molecular clock to ND2 and C-myc2, and relaxed lognormal clock to β-fibint7 and CXCR4. We fitted the substitution rate with the ND2 calibration as in IMa2 analysis (see above). To estimate the prior for the mean distribution of population sizes we performed preliminary analysis as suggested by the authors (results not shown). We started by conducting an mtDNA run with a coalescent prior and constant population size, and then we used the obtained mean value (0.13) as prior in the final run with a log-normal distribution and standard deviation of 0.5. We performed three replicate runs with 100 million generations, with trees being sampled every 10,000 generations and the first 10% of the samples being discarded as burn-in. We checked convergence through ESS values (>200) in the software Tracer 1.5 (available at: http://tree.bio.ed.ac.uk/software/tracer/). For results plot and interpretation, we used Python scripts provided by the authors (available at: http://beast.bio.ed.ac.uk/Tutorials), assuming a generation time of 1 year.

Testing explicit scenarios of diversification

For a more robust biogeographic inference, we performed a multilocus ABC analysis testing which of a set of concurrent scenarios best fit the diversification history of *P. distincta*. We designed four plausible scenarios by gathering information on the habitat and studied system (e.g. Thomé *et al*., 2014), including suggestions of putative population structure based in mitochondrial data from previous work (Brunes *et al*., 2010), demographic changes expected under proposed LGM forest dynamics (Carnaval & Moritz, 2008), and on the results of the demographic analyses conducted previously in this paper (population expansion and gene flow). The four scenarios (Fig. 4.2) were as follows: A) a null hypothesis of absence of structure combined with little or no habitat fluctuation, where a single population show long-term persistence possibly followed by moderate Holocene expansion ('long-term persistence'); B) a combination of the effects of a barrier and little habitat fluctuation,

in a scenario where two populations were formed by an ancient vicariant event possibly followed by moderate Pleistocene retraction and Holocene expansion ('ancient vicariance'); C) a scenario where population structure is driven by recent habitat fragmentation, with an ancestral population splitting into two geographically restricted populations during the LGM, followed by strong Holocene expansion ('two refugia'), and D) a scenario where the formation of two populations occurs through the recent (post LGM) colonization of a formerly inhabited region by migrants from a stable population (e.g. Carnaval *et al*., 2009) ('recent colonization').

For each scenario we simulated equal numbers of genealogies, selected the simulations that best fitted the observed data with the aid of summary statistics (rejection step), and checked for the fit of accepted genealogies. We interpreted the proportion of selected genealogies from each concurrent scenario as a direct measure of its posterior probability (see Pritchard *et al*., 2000), and calculated the Bayes factor to access inference power (Jeffreys, 1961). The parameters included in each scenario were: ancestral population size, divergence time, population size of each subpopulation, migration, and exponential growth (g=-(1/time)*log(theta1/theta2)). The priors for total population size and divergence time were provided per locus. For total population size we first ran a short analysis with broad priors (0.1 - 50) sampling values from uniform distributions. After the rejection step, we extracted mean values and standard deviations from the posterior to be used as Gaussian priors for the definitive analysis (ND2, mean=13.01/SD=11.69; β-fibint7, mean=12.22/SD= 9.35; and CXCR4, mean= 6.51/SD= 5.84). For the divergence time in scenario B, we extracted mean values from IMa2. Because the right tail of the approximate posterior density curve failed to reach zero (see Results), we arbitrarily chose large intervals (SD=300,000 years). For LGM divergences (scenarios C and D) and growth duration (all scenarios), we used a mean of 21,000 years (SD=10,000 years). For all other parameters, we used uniform priors, with the following intervals: subpopulation sizes varied from 0.4 to 0.6 of total population size, migration varied from zero to the maximum of the 95% highest posterior density interval in IMa2 analyses, and growth varied according to the scenarios. In long-term persistence and ancient vicariance (scenarios A and B) we set a growth ratio interval of 0.60–1 (60–100% of current population sizes remained prior to demographic expansion), which allows from moderate bottlenecks to total stability. For the two refuge scenario we set a growth ratio of 0.01–0.20 for both populations, simulating

more drastic demographic reductions (only 1–20% of their current effective size remained prior to demographic expansion), whereas for the recent colonization we kept 0.60–1 for the first population (Northern) and 0.01–0.20 for the second population (Southern).

We generated 1,000,000 genealogies per locus for the definitive analysis (100,000 for prior selection) through the program ms (Hudson, 2002), for which we obtained diversity statistics with a modified version of SampleStats (Hudson, 2002). We automated this process using perl scripts (Appendix S1). After preliminary analyses, we removed the marker C-myc2 to avoid unrealistic results due the low number of gene copies and low variability. We performed the rejection step in msReject (Hickerson *et al*., 2007) with a tolerance of 0.0001 (0.01 for prior selection). Following Pelletier & Carstens (2014) we used three distinct measures of nucleotide diversity (within population one, within population two, and between populations) as observed summary statistics, which were calculated on a per locus basis in DnaSP 5.0 (Librado & Rozas, 2009). Finally, we performed the rejection step both jointly and per locus, evaluating for model fit using principal component analysis (PCA) of the diversity statistics to check for dispersion away from observed values. Therewith, we expect that a central position of the score of the observed data relative to the distribution of scores from the selected simulated genealogies in the ordination plots presenting a high goodness of fit.

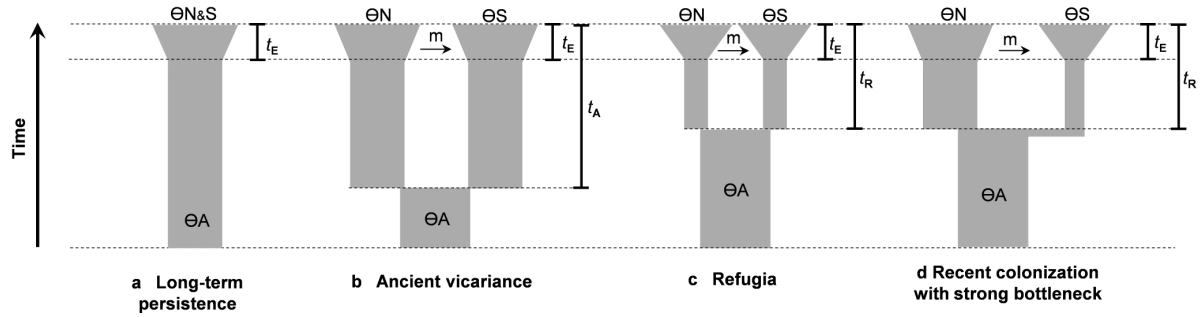

Fig. 4.2. Possible demographic models of the diversification of *Phyllomedusa distincta* in the Southern Brazilian Atlantic Forest used in the approximate Bayesian computation analysis. Model parameters: θ = 4Neμ of ancestral (A), North (N) and South (S) groups; 2Nm = m with arrow indicating direction; T_A = ancient divergence time; T_R = refugia divergence time during the Last Glacial Maximum; T_E = timing of expansion starting in the Holocene going in thru the present.

Bioclimatic models

To evaluate if climatic suitability areas for our focal taxa were stable during the Last Interglacial period (120 ka, LIG) and, subsequently, experienced drastic range reductions during the Last Glacial Maximum (21 ka, LGM), (following theoretical

expectations from the refuges theory, (Haffer, 1997), we generated correlative maps of the past and current potential distribution of *Phyllomedusa distincta* through the maximum entropy algorithm implemented by MaxEnt 3.3.3k (Phillips et al., 2006). MaxEnt's method has a positive established trajectory been widely used in SDM's combined with phylogeographic studies (Elith *et al*., 2006, 2011, and therein references). We used 31 occurrence points (Table 4.1 and Fig. 4.1) and the climatic layers available in the WorldClim database at ~5 km^2 spatial resolution (Hijmans *et al*., 2005). After preliminary evaluations (see below), we projected the current potential distribution for the LIG (120 ka) and for the LGM (21 ka), both models provided by the Community Climate System Model (CCSM) (Otto-Bliesner *et al*., 2006, 2007). Following Anderson & Raza (2010), we calibrated the models in a defined study area. We used a buffer of 300km from the current range distribution (endemic of the Southern Ombrophylous forest) of *P. distincta* (Fig. 4.6a). We selected five out of 19 bioclimatic variables with a Pearson correlation lower than 0.85 (data not shown) under current climatic conditions. The selected variables included: Mean Diurnal Range – BIO2, Temperature Seasonality – BIO4, Annual Precipitation – BIO12, Precipitation Seasonality – BIO15, Precipitation of Warmest Quarter – BIO18. We estimated the contribution of each variable to explain the potential species distribution by performing a jackknife test.

Following recent evidence that species-specific tuning can improve the performance of MaxEnt models (Anderson & Gonzalez, 2011; Radosavljevic & Anderson, 2013), we varied the regularization multiplier values (0.25, 0.50, 1.00, 1.50, 2.00, 4.00, 6.00, 8.00 and 10.00) to prevent overfitting. We performed statistical analysis with random test percentage of occurrence points (75% for model prediction, and 25% for model validation) and 10 replicates. We based quantitative model evaluation on the threshold-independent Receiver Operating Characteristics Curve (ROC) identifying the lowest values of average differences between training and test area under the curve (AUC). In addition, we also evaluated the model performance via i) qualitative visual inspections of the response curves looking for smoother and regular patterns (e.g. Camargo *et al*., 2013), ii) the predictive maps compared to expert knowledge of the distribution of BAF phytophysiognomies (IBGE, 2012) and the known distribution of *P. distincta* (Haddad *et al*., 2008). We then used the best parameter set to generate the current and past climate projections of *P. distincta*. The final maps are presented with the original probability distribution generated by

MaxEnt, considering the gradual changes from suitable to unsuitable habitats in nature. We avoided the use of an arbitrary threshold to produce binary maps of species presence/absence because thresholds may be subjective and constitute an additional source of error in ecological niche modelling (Liu *et al*., 2005; Phillips *et al*., 2006).

4.1.4 Results

Phylogeographic and population structure

Single gene haplotype genealogies presented patterns that differ between mitochondrial and nuclear fragments (Figs. 4.1b and Fig. C.1). The mtDNA ND2 haplotype genealogy showed two highly divergent clades (*p-uncorrected*=1.5%), with one grouping most of samples located in São Paulo and Paraná states (Northern lineage), and another grouping all individuals from Santa Catarina and Rio Grande do Sul states (Southern lineage). Shared haplotypes were found in only one locality on an intermediate geographic location (SL 14; one haplotype from the Northern lineage and seven from the Southern lineage). Haplotypes from the Northern lineage are slightly structured while the Southern lineage showed a star-like topology, with the exception of one unique internal haplotype from Apiúna (SL19; Fig. 4.1b). The structure in nuclear fragments is not as geographically well defined, with all nuclear haplotype genealogies sharing variation between the two lineages defined by the mtDNA topology. Shared variation was less evident in C-myc2 and β-fibint7 than in CXCR4 as genealogies of these markers showed a tendency to separate haplotypes from both lineages. The β-fibint7 genealogy also presented some external haplotypes in the Southern region (SL 17, 18, 19, 23, 30) that grouped with the Northern ones. The group formed by the remaining haplotypes from the Southern region showed less genetic variation when compared with the Northern ones, resembling the pattern in the mtDNA structure.

When all the nuclear locus were jointly analyzed, POFAD showed a strong tendency to separate individuals from the Northern and Southern region with the presence of some intermediates (SL 18, 19-20; Fig. 4.3a). The STRUCTURE analysis recovered two clusters (K=2), according to the distribution of Ln Pr (X/K) and ΔK (Fig. C.2), with geographic distributions that also form a Northern and a Southern group (Fig. 4.3b). While the Northern cluster showed high exclusive values of

assignments, the Southern cluster presented some intermediate individuals (SL 18-20, 23, and 30; ~Q values of 0.33–0.67) and one entirely allocated to the opposite cluster (SL 19).

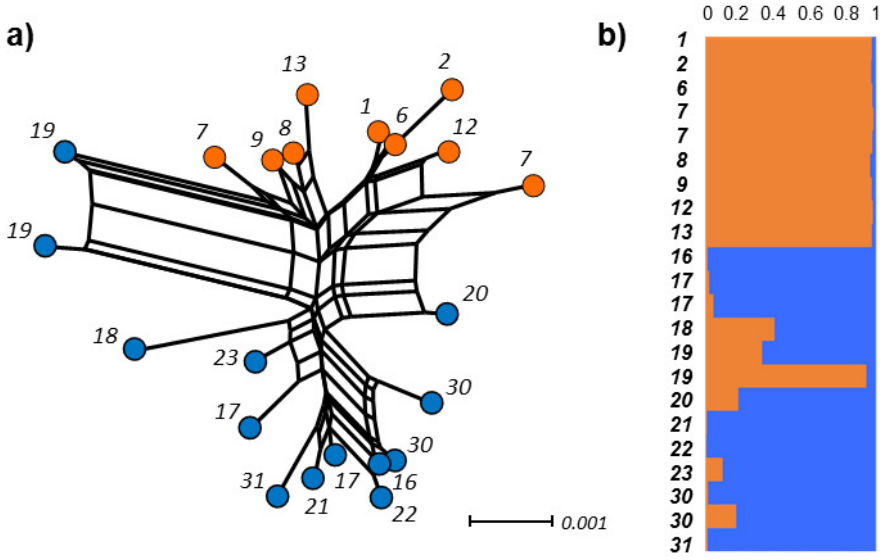

Fig. 4.3 Multilocus nuclear structure of *Phyllomedusa distincta* based on the three nuclear fragments (CXCR4, β-fibint7, C-myc2): a) genetic distance network; circles were colored following the two major mitochondrial groups (see Fig. 4.1); and b) STRUCTURE results based on nuclear allele frequencies for K=2. Individuals are represented as bars, with colors representing the proportion of assignment. Sampling localities are presented near of circles and left of each bar (see Table 4.1 and Fig. 4.1).

Genetic variation

Levels of polymorphism in mitochondrial and nuclear fragments showed substantial variation (Table 4.2). The 993bp ND2 fragment revealed 17 haplotypes defined by 28 segregating sites. The total nuclear DNA variation based in each fragments revealed 26 (β-fibint7), 10 (CXCR4), and 4 (C-myc2) haplotypes defined by 20, 10, and 5 segregating sites, respectively. The highest nucleotide diversity was found in the ND2 fragment (0.75%) and the lowest in CXCR4 (0.26%). Both ND2 and β-fibint7 showed higher values of Θw (0.53 and 0.56%, respectively), followed by C-myc2 and CXCR4 (with 0.45% and 0.35%). Comparing both groups and locus, nucleotide diversity was higher in Northern group than in Southern group, with the exception of the CXCR4. For the ND2 fragment, the lowest nucleotide diversity (0.03%) was observed in the Southern group (ND2; 95% CI 0–0.11%) representing the variation a total of 71 individuals. Comparatively, nucleotide diversity in the Northern was ~5-fold higher (0.16%; 95% CI 0.02–0.43). We also detected an indel of 4 and 5 bp-long in β-fibint7 and C-myc2, respectively. The DSS test did not detect any recombination left in any of the nuclear fragments after phasing. Due to the low number of individuals analyzed here summary statistics of C-myc2 should be interpreted with caution.

Table 4.2 Fragment information, summary statistics, and neutrality tests within the main mitochondrial clades and nuclear groups (see POFAD results) of *Phyllomedusa distincta*.

Fragment	Groups	Length	Polymorphisms							Neutrality tests		
			N	SS	Sn	h	% Hd	% θ [95% C.I.]	π [95% C.I.]	Tajima's D	Fu's Fs	R_2
ND2	All	993	118	28	10	17	66	0.53 [0.41-1.26]	0.75 [0.25-1.72]	-	-	-
	North		47	9	4	9	72	0.2 [0.04-0.34]	0.16 [0.02-0.43]	-0.6026 ns	-2.1381 ns	0.0885 ns
	South		71	9	9	8	19	0.19 [0-0.08]	0.03 [0-0.11]	-2.2743 ***	-9.4589***	0.0491 ns
β-fibint7	All	600-604	102	20	6	26	86	0.56 [0.25-1]	0.56 [0.14-1.36]	-	-	-
	North		60	15	5	12	75	0.53 [0.18-0.85]	0.45 [0.1-1.14]	-0.4381 ns	-1.6585 ns	0.0903 ns
	South		42	12	1	15	70	0.46 [0.11-0.76]	0.38 [0.07-0.96]	-0.5149 ns	-6.7597*	0.0955 ns
CXCR4	All	609	62	10	2	10	78	0.35 [0.07-0.52]	0.26 [0.03-0.69]	-	-	-
	North		26	3	2	4	44	0.13 [0-0.26]	0.09 [0-0.29]	-0.8212 ns	-1.2269 ns	0.1081 ns
	South		36	8	2	8	83	0.32 [0.08-0.71]	0.34 [0.05-0.88]	0.1187 ns	-1.6195 ns	0.1125 ns
C-myc2	All	318-323	18	5	1	4	65	0.45 [0.09-1.08]	0.44 [0.03-1.24]	-	-	-
	North		12	4	1	3	53	0.41 [0-1.13]	0.45 [0-1.28]	0.3853 ns	1.5263 ns	0.1770 ns
	South		6	1	0	2	53	0.14 [0-0.54]	0.16 [0-0.59]	0.8505 ns	0.6254 ns	0.2666 ns

* P < 0.05 *** P < 0.001 ns = no significance

Divergence time, ancestral population parameters and gene flow estimates

Analyses using the Isolation-with-Migration model showed posterior distribution of parameter estimates consistent across replicate runs with effective sample sizes (ESSs) values>120. In two cases (divergence time and theta) the right tail of the approximate posterior density curves (Fig. C.3) failed to reach zero. Nevertheless, these analyzes suggested the divergence between Northern and Southern groups occurred at ~0.61 Myr, during the Pleistocene. Estimates of effective population size for both groups were very similar (Ne ~$2.6x10^5$, 95% HPD ~$1.2x10^5$ – $4.1x10^5$). The 95% HPD of the ancestral effective population size starts in the upper limit of the current estimates ($9x10^5$, 95% HPD: $4.1x10^5$ – $1.3x10^6$) suggesting that both groups experienced a reduction of the effective population size at some period of time. Estimates of gene flow showed an asymmetrical result, detecting migrant gene copies only from the Northern to the Southern group (2nm = 0.7, 95% HPD: 0 – 4, LLR: 5.623, P < 0.01).

Demographic history

Deviations from the neutral theory (Table 4.2) were significant only in the Southern group, as observed for ND2 (Tajima's D and Fu's *Fs*) and β-fibint7 (Fu's *Fs*). Although the R_2 test did not show a significant *p*-value for the putative expansion of the Southern group as in others tests, values were lower in the Southern group than in Northern, for the ND2 fragment. The multilocus EBSP analysis of historical

demography over time, showed a trend of smooth and gradual population increase for the Northern group starting around the early Holocene according to the median (Fig. 4.4a). Nearly at the same time, the Southern group started an abrupt population growth with a 10-fold increase in effective population size, (Fig. 4.4b). Confidence intervals for both analyses are large and include the possibility of no growth.

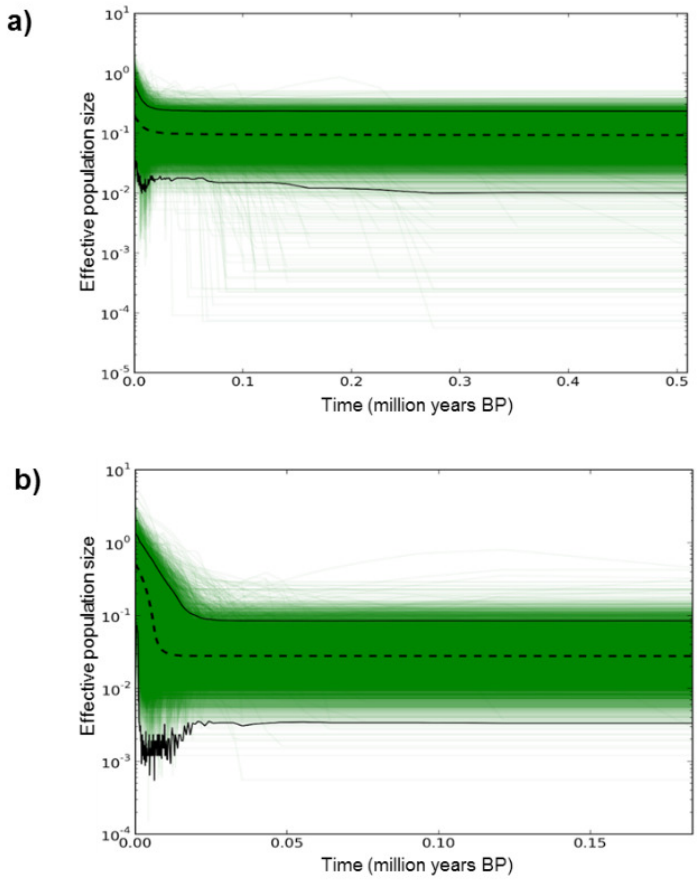

Fig. 4.4 Extended Bayesian Skyline plots of *Phyllomedusa distincta* groups: a) North group, and b) Southern group. Dashed line represents the median population size (multiply by thousand and by generation time of one year), and the lightly shaded grey the 95% HPD. The accumulation of green lines represents the full posterior distribution. The Y axis are in logarithmic scale.

Testing explicit scenarios of diversification

The scores representing the vectors of the summary statistics calculated from the observed data were central to the vectors of the simulated genealogies (Fig. 4.5) indicating a good fit of the models to the data for ABC analyses. After the rejection step, scenarios A (one population with long-term persistence) and D (recent colonization) received zero or very low posterior probability values in both per locus and multilocus analyses. The posterior probabilities of the models were congruent between β-fibint7 and CXCR4, supporting the ancient vicariance scenario (Fig. 4.2b), whereas, ND2 rejection was inconclusive, presenting similar support values to

scenario B (Ancient vicariance) and C (Refugia). Multilocus rejection were decisive, supporting the scenario B with substantial support according to the Bayes factor (Table 4.3).

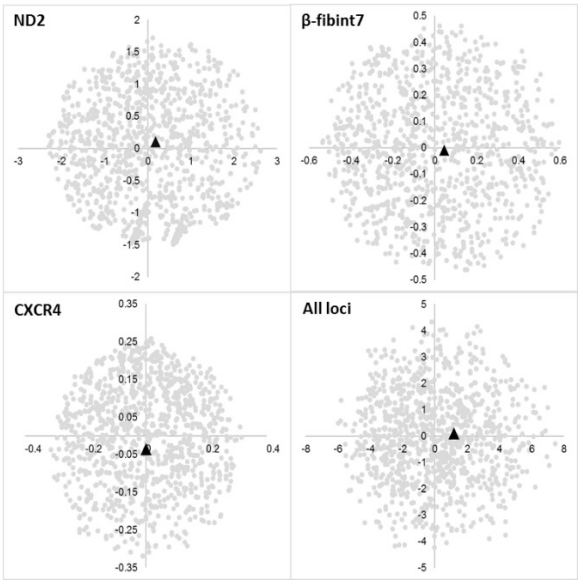

Fig. 4.5 Model checking in ABC analyses of *Phyllomedusa distincta*. Ordination plots show summary statistics vectors of simulated (grey circles) and observed (black triangles) data after the rejection step. We conducted the rejection step per locus and for all loci conjunctly (see text for details).

Table 4.3. Posterior probabilities and model support (Bayes factor) of historical demographic models of *Phyllomedusa distincta* assessed by an approximate Bayesian computation analysis (see Fig. 4.2). The rejection step was performed per locus and for tree loci together.

Scenarios	ND2	β-fibint7	CXCR4	All loci
Long-term persistence	0	0	0.08	0
Ancient vicariance	0.40	0.74	0.55	0.86
Refugia	0.59	0.18	0.31	0.13
Recent colonization	0.01	0.08	0.06	0.01
K	1.5	4.1	1.8	6.6
Bayes factor	Barely worth mentioning	Substantial	Barely worth mentioning	Substantial

Bioclimatic models

The species-specific tuning results showed a slight quantitative variation, but a wide range of qualitative variation according with the regularization parameter used (Table C.2). The average test AUC values were high (0.88-0.98) and the difference between training and test AUC varied of the -0.019 to 0.04. Overall, the average omission rates based on the MTP test showed the lowest and more constant values with the regularization multiplier set to 1.0, similar to Radosavljevic & Anderson (2013). Contrary to expectations, the species-specific tuning of *P. distincta* presented regular/smoother response curves and more realistic predictive suitability areas using

the regularization parameter set to 1.0 (see Radosavljevic and Anderson 2013). Based on these results, the default regularization multiplier (1.0) was selected (see Table C.2 and Fig. C.4 to response curves). The jackknife test identified the most important climatic variables to explain the distribution of *P. distincta* in the BAF (Fig. C.5). Of the five climatic variables used, the mean diurnal range (BIO 2), the temperature seasonality (BIO 4), and the precipitation seasonality (BIO 15) presented smaller differences both in gain (test and training) and in AUC values.

The present time predictive map showed an area with high climatic suitability compatible with the current distribution of the *P. distincta*. Areas of overprediction appeared in central regions of Rio Grande do Sul and Rio de Janeiro states (Fig. 4.6a). Similar overpredictions were observed in both paleoclimatic projections (Fig. 4.6c). As expected, the present time and the LIG projections yielded similar distribution maps. The LGM projection predicted intense fragmentation with two small isolated areas within the current distribution of the *P. distincta*, one in the current coastal region of Paraná state reaching to the north of Santa Catarina state, and another located in the limit of the LGM land masses in central Santa Catarina state (the sea level was lower than today).

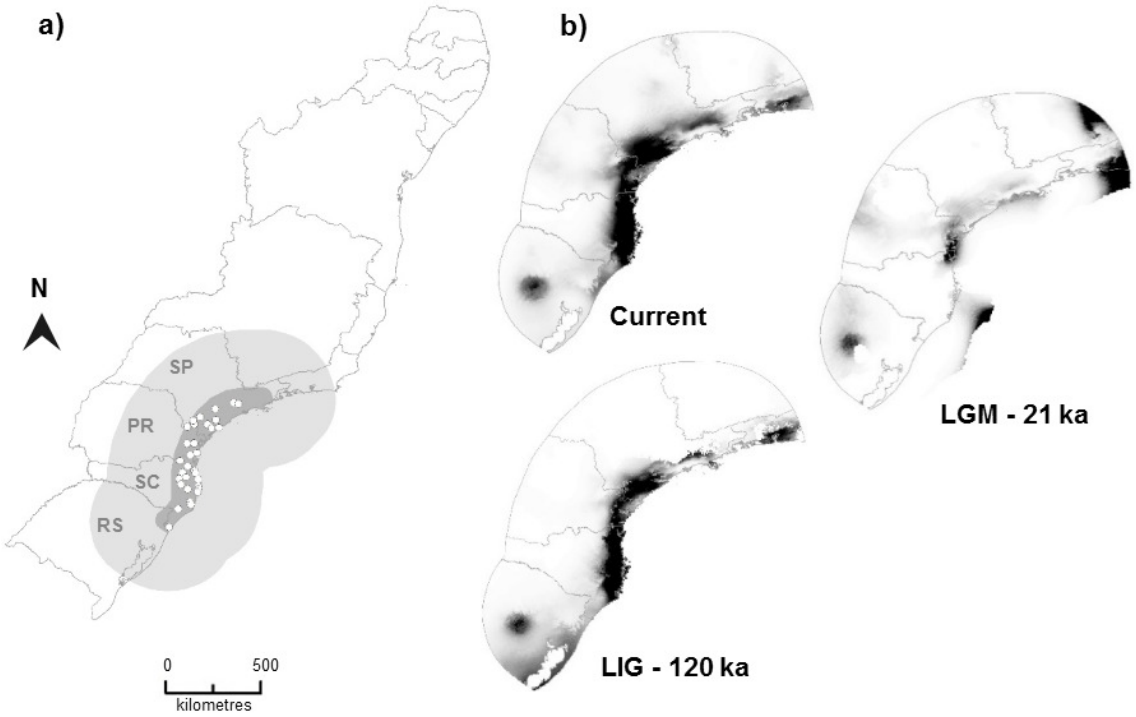

Fig. 4.6 Maxent models for *Phyllomedusa distincta* in southeastern Brazilian Atlantic forest: (a) study area in grayscale (see text for details); and (b) predicted climatically suitable areas for current time, Last Glacial Maximum period (LGM), and Last Interglacial period (LIG). Dark colors represent high climatic suitability and soft colors represent low climatic suitability. Both paleoclimatic models were performed under the Community Climate System Model (CCSM). White dots indicate occurrence points used in the analysis. Brazilian states: SP, São Paulo; PR, Paraná; SC, Santa Catarina; and RS, Rio Grande do Sul.

4.1.5 Discussion

It is well demonstrated that phylogeographical investigations require extensive geographic sampling and multilocus analysis, and that they may be strengthened by using complementary paleomodeling of species distributions. In this study, we were able to clarify the evolutionary history of *Phyllomedusa distincta*, an endemic leaf frog species from the Southern Brazilian Atlantic forest. Overall, our results indicate the presence of two divergent lineages with coherent geographic distribution within *P. distincta* range. Demographic estimates (from basic statistics, neutrality tests and ESBP) suggested that the Southern lineage has experienced a recent population expansion that started in the Holocene. The testing of alternative historical demographic models (ABC analysis) favored a scenario of ancient (mid-Pleistocene) vicariance with moderate Holocene population expansions, whereas paleodistribution modelling inferred past habitat fragmentation, corroborating the signals of recent demographic expansion for the South lineage. Comparing our results to findings from different co-distributed organisms, we provide a broader perspective of species diversification in southeastern BAF.

Mitochondrial and nuclear structure

The mtDNA ND2 haplotype genealogy confirmed the early findings of Brunes *et al.* (2010) in revealing the presence of two highly divergent lineages (*p-uncorrected*=1.5%) with coherent geographic distribution within the *P. distincta* range. The drastic sampling increase relative to a previous study enabled us to detect one locality with the presence of haplotypes from both mtDNA lineages, which may indicate gene flow between lineages. In general, single locus nuclear genealogies did not recover straightforwardly the two lineages observed for mtDNA, which could be explained by the retention of ancestral polymorphism and/or gene flow. Such incongruence between cytoplasmic and nuclear gene genealogies is expected due the higher effective population sizes and lower mutation rates of nuclear loci as previously demonstrated by Edwards & Beerli (2000). However, multilocus approaches based on the coalescent theory and/or allele frequency–based have been successful in combining gene genealogies to reveal population groups even with reduced genome sampling and in the presence of incomplete gene lineage sorting and hybridization (Thomé *et al.*, 2012; Fusinatto *et al.*, 2013). Here, the two

lineages of *P. distincta* revealed by mtDNA analysis were unambiguously corroborated by multilocus analyses, albeit with some evidence of intermediate genomes suggesting either incomplete lineage sorting or mixing. Whereas ancestral polymorphism is necessarily present at some time window along the divergence process itself, gene flow may or may not occur along the evolutionary scenario that generates deep divergence between populations. For the data presented here, the distinction between the processes was better investigated through multilocus models of isolation in the presence of gene flow and hypothesis testing of alternative historical demographic scenarios (IMa and ABC, see below).

Divergence, gene flow and demography

The isolation with migration model implemented in IMa inferred the mean divergence time between Northern and Southern group to be ~600,000 years, well into the Pleistocene. The boundary between the two diverged groups geographically coincides in the present day with the Cubatão shear zone, listed as tectonic fault with Quaternary surface rupture (Saadi *et al*., 2002). No information is however available on the timing and extent of ruptures in this geological complex. Migration from Northern into Southern was much higher than in the opposite direction. This result could be perhaps associated to higher demographic stability inferred for the Northern group but to adequately address the hypothesis of asymmetric gene flow would require a detailed description of the contact zone which is not in the scope of this study.

Multilocus estimates of demographical change across time suggest distinct histories for the two groups of populations of *P. distincta* in southern BAF. EBSP for both groups show large confidence intervals, but also depicts trends of recent growth that seem to vary in intensity. Populations in the southern range of *P. distincta* (Southern group) may have experienced higher demographic instability than populations in the northern parts of the range (Northern group). Evidence from other analyses are in accordance with this result, suggesting at least some expansion for the Southern group as observed for ND2 (Tajima's D and Fu's *Fs*) and β-fibint7 (Fu's *Fs*). This result reflects the overall pattern of high values of segregating sites and haplotypes when compared with the nucleotide diversity. Also, comparing both groups and locus, nucleotide diversity was higher in Northern group than in Southern

group for most fragments, with the exception of the CXCR4, as expected by the pattern of incomplete lineage sorting found in this fragment. The inconsistence of some diversity statistics across loci seems to reflect differences in mutation rates, as 'faster' markers such as mtDNA and introns showed significant values more often, while differences between tests may be also explained by the better performance of Fu's *Fs* test with larger sample sizes (see Ramos-Onsins & Rozas 2002).

Our results are partially concordant to studies reporting on the demographics of other southern BAF taxa. In southern BAF, populations of some organisms were shown to have experienced more demographic instability than northern populations (treefrogs of the genus Hypsiboas, Carnaval *et al*. (2009); antbirds of the genus Myrmeciza, Amaral *et al*. (2013: mammals of the genus Akodon (Valdez & D'Elía, 2013), while higher demographic stability was inferred for the southern populations of toads of the Rhinella crucifer group (Thomé *et al*., 2014). It is difficult however to exactly compare between these studies because the population boundaries are not the same. Most studies have revealed population boundaries to be in the central or southern state of São Paulo, while the genetic boundary here described is the first located in the more southern transition between the states of Paraná and Santa Catarina.

Testing explicit scenarios of diversification

Multilocus data and multidisciplinary approaches has been considered of major importance to design and test hypotheses exploring the evolutionary history of BAF organisms due its geological/climatic complexity and scarcity of independent evidence (Amaral *et al*., 2013; Thomé *et al*., 2014). Here, we used multilocus data in an ABC framework to test for alternative diversification scenarios within the leaf frog species *P. distincta*. Our simulations showed a good fit to the observed data, demonstrating that acceptance of models was not a result of choosing the best among a set of poorly fitting scenarios.

Very low (or null) posterior probabilities both for the rejection performed per locus and jointly allowed us to clearly reject two scenarios for the evolution of *P. distincta* populations: A) the long-term persistence of one single demographically stable population (Fig. 4.2a and d) and the recent colonization of southern areas of the range from northern populations (Fig. 4.2d). Two other scenarios could not be

readily rejected as explanatory for the genetic diversity observed within *P. distincta*.

The ancient divergence of two relatively stable populations (Fig. 4.2b) received substantial support from the joint locus rejection and from the β-fibint7 data. On the other hand, the scenario of LGM divergence following severe bottleneck effects resulting from habitat fragmentation followed by Holocene demographic expansions (Fig. 4.2c) should not be completely ruled out because it had as much support as hypothesis A from the mtDNA data analysis. Despite of this, the highest posterior probability obtained for joint locus rejection favors the ancient vicariance as the best fitting scenario for *P. distincta* evolutionary history.

Paleoclimatic distribution modeling

Paleoclimatic distribution modeling of *P. distincta* suggested that there was a dramatic reduction of suitable areas for the species occurrence during the LGM. Within the current distribution of *P. distincta*, one single area in coastal Paraná state would represent a refuge as the other putative area of LGM species persistence is today covered by the Atlantic Ocean. This result is not completely at odds with the findings from our genetic analyses. Rather, there is a geographic overlap of the single remaining area in coastal Paraná state and the distribution of the Northern lineage, which is also more according to EBSP and neutrality tests. However, our paleomodeling results hardly predicts any habitat left for the Southern lineage during the LGM, which would imply a scenario of recent colonization that was formerly rejected in ABC. Thus, here we believe that the paleomodeling results are showing support to the impact of late Pleistocene climatic oscillations on demography of *P. distincta* (e.g. Álvarez-Presas *et al*., 2014), in particular, on populations from the Southern lineage where its location seems to have been much more instable than the Northern ones. Nevertheless, we cannot exclude the possibility that our analysis was affected by the coarse ability of species distribution paleomodeling to deliver detailed predictions of historical distributions in the South America region (see Rojas *et al.* 2009 and Collevatti *et al.* 2013).

The evolutionary importance of the southern BAF region (including some southern areas) in shaping the present-day biodiversity has been highlighted since the end of the seventies based on different types of data (Porto *et al*., 2013 and therein references). Interestingly, our result runs parallel with recent findings of

Carnaval *et al*. (2014) based on modeling partitioned-BAF analysis. The potential LGM coastal refugia in Paraná state found here was recently considered an area with higher values of phylogeographic endemism (Carnaval *et al*., 2014). Data from pollen cores and soil chemical isotope analyses represent a more realistic, albeit spatially discontinuous, source of paleoecological evidence. Palynological evidence suggests that cooler and dryer climates had an effect of reducing semideciduous forests in the southern of BAF (e.g. Behling, 2002), but *P. distincta* only occurs in the non-seasonal coastal and sub-montane rainforests. Data from chemical isotopes in speleothems located in non-seasonal rainforest areas suggests otherwise that the LGM climate may have been as wet as in the present (Cruz *et al*., 2005). Much more data will be needed to better understand what paleoecological conditions southern BAF species may have endured since the LGM.

Patterns of diversification in southern Brazilian Atlantic forest

There is an ongoing debate on the relative contribution of geographic barriers and Pleistocene refuges in determining diversification of forest dependent taxa in the BAF (e.g. Carnaval *et al.* 2009; Thomé *et al.* 2010; Amaral *et al.* 2013). The challenge is now to undertake studies designed to distinguish between the alternative, but non-exclusive, forest refugia and barrier hypotheses to explain diversification patterns of BAF organisms. A previous account of the diversification patterns in the *P. burmeisteri* group throughout the BAF (Brunes *et al*., 2010) suggested that both the Tertiary and Quaternary were important for the diversification of species within the group, a result in agreement with other molecular studies in BAF organisms (e.g. Grazziotin *et al.* 2006; Thomé *et al.* 2010; Amaral *et al.* 2013). Phylogeographic breaks across the BAF have been shown to coincide mainly with major rivers (Doce river) and/or regions with recent neotectonic phenomena such as the Guapiara lineament near Ribeira de Iguape in southern state of São Paulo (e.g. Saadi *et al*., 2002; Ribeiro, 2006; Thomé *et al*., 2010). In this study we report the divergence between two population groups of the species *P. distincta* in the southern BAF, which presently meet near the southern end of the Cubatão shear zone (see Saadi *et al*., 2002). We distinguished between refugial and barrier hypothesis to explain population divergence, by contrasting alternative demographic scenarios involving the vicariance between populations undergoing extensive demographic changes

across time (refugia) or the vicariance between more or less stable populations (barrier), and concluded that generally vicariance has undergone without substantial demographic change at least since the LGM, but that demographic change cannot be completely ruled out. It is therefore not yet clear whether the Cubatão shear zone has been a stable primary barrier or a zone of secondary contact resulting from Holocene range expansions from northern and southern refugial areas. However, it is worth mentioning that recently multilocus ABC analyses of a BAF endemic toad reinforced the whole of geographic barriers in promoting the main divergences in *Rhinella crucifer* complex, with refugia presenting a secondary role by causing intraspecific structure (Thomé *et al*., 2014).

CONCLUSIONS

The southern end of the Cubatão shear zone, near the transitions between the states of Paraná and Santa Catarina, has been for the first time associated to a phylogeographic break in the BAF. This pattern adds to the mosaic that is arising from studies of diversification in several BAF organisms, replacing the elegant simplicity of the Carnaval *et al.* (2009) hypothesis with idiosyncratic complexity (see Thomé *et al*., 2010). The recent increase in the number of phylogeographic studies in the BAF associated to apparently idiosyncratic patterns argues for the need of a first meta-analysis following a rigorous hypothesis testing approach that may provisionally clarify the relative importance of refugia *versus* barriers in shaping lineage and species diversity in the megadiverse BAF.

4.1.6 Acknowledgments

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DATA ACCESSIBILITY

A table with information on specimens (individual information and GenBank accession numbers), alignments for all loci and ABC scripts are available at Dryad Digital Repository.

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Capítulo 5

Diploide *versus* poliploide: comparações com base em Sistemas de Informação Geográfica

5.1 Distinct spatial and environmental distributions in diploid-polyploid Neotropical leaf frogs[6]

5.1.1 Abstract

Aim Polyploidy distributions have been associated with climatically unstable regions and harsher habitats. Here, we test this hypothesis comparing niche environmental characteristics of Neotropical amphibian populations of tetraploid (*Phyllomedusa tetraploidea*) and phylogenetically close diploid species (*P. distincta* and *P. iheringii*), and identify key factors related with the maintenance and position of putative sympatric areas.

Location South-eastern South America

Methods We analysed 70 georeferenced occurrence points with a set of climatic, topographical, and habitat variables (1×1 km grid cell), representative of the whole distribution range of each species, using ecological modelling (Maxent), multivariate statistics (PCA), and niche comparisons (ENMtools).

Results Maxent analyses suggest that all distributions are more related with climatic than with habitat factors. For all diploid-diploid and diploid-polyploid niche comparisons, our results were congruent in showing that the more divergent niche is between the diploid *P. iheringii* and the polyploid *P. tetraploidea*. The polyploid species tend to occur in areas of high thermal amplitude and high potential evapo-transpiration when compared with both diploid species. Two putative sympatric areas were predicted, both mirroring patterns of close environmental similarities between diploid-diploid and diploid (*P. distincta*)-polyploid species. The fine-scaled analysis of both areas suggests that only the previously described natural hybrid zone between *P. tetraploidea* and *P. distincta* is located in an area with smooth climatic transition.

Main conclusions The polyploid species occupies distinct ecological conditions compared to the two diploid relatives, being coincident with areas of harsher climate. Predicted sympatry corroborates previously location of a hybrid zone between *P. distincta* and *P. tetraploidea*, and suggests an extremely reduced chance of hybridization between diploid species. GIS-based analyses performed at a fine-scale

6 This work is in preparation for publication: Brunes TO, Brito JC, Alexandrino J, Haddad, CFB & Sequeira F.

level can be useful to address polyploid origin, their ecological capability, and factors affecting location and maintenance of natural hybrid zones between different ploidy levels. Globally, this approach could be useful for future comparative studies in determining the role of biogeography in polyploid origin, evolution, and distribution.

5.1.2 Introduction

Polyploidization is regarded as a particular mode of instantaneous speciation event whereby the newly formed polyploid immediately becomes reproductively isolated from progenitor species. Traditionally, polyploids are classified either as allopolyploids, derived from the combination of distinct genomes through hybridization or autopolyploids, through genome duplication of a single species (Stebbins, 1950; Coyne & Orr, 2004). Although polyploidy has been widely accepted as an important evolutionary force in plants, its role as driver of evolutionary adaptation and/or diversification in animals is still poorly known. Nevertheless, polyploidy also occurs in animals, being particularly common in ectothermic vertebrates such as fishes, reptiles, and amphibians (Mable *et al.* 2011, and therein references). Indeed, the growing body of literature in recent years has highlighted the importance of this phenomenon as a prominent force shaping the evolution of eukaryotes, particularly as promoter of adaptive evolutionary changes (Otto & Whitton 2000; Mable 2004; Osborn *et al*. 2007).

Despite of this, key questions related with the establishment of polyploids are still intriguing, particularly how newly formed polyploids overcame the mating disadvantage because of the minority citotype (i.e. minority citotype exclusion; Levin, 1975). Theoretical predictions invoke competitive replacement of diploid progenitors and/or ecological differentiation, including the colonization of new habitats following periods of climatic unstable conditions (e.g. Otto 2007; Mable *et al.* 2011, Petit *et al*. 1999; Ramsey & Schemske 2002). The classical view is that polyploids are more tolerant and able to colonize harsher environments than their diploid counterparts due to changes in physiology, resulting from both increased cell size and genetic background (heterosis and gene redundancy) provided by having at least one more copy of the genome. The observation of a higher frequency of polyploids in unstable climatic areas, corresponding to severe environmental conditions (Stebbins, 1950; Levin, 1983; Soltis & Soltis, 1999) reinforces this classical view. However, recent qualitative and quantitative studies based on Geographical Information System (GIS),

found that the association of polyploidy with harsher environments and broader distribution ranges relative to their diploid counterparts appears not to be a universal outcome, but seems to be species-specific (Martin & Husband 2009; Mable *et al*. 2011; te Beest *et al*. 2012; Theodoridis *et al*. 2013). Notwithstanding, these results were predominantly based on plants, and few quantitative data is available for animal systems (see Otto *et al*. 2007).

The Neotropical species of leaf frogs from the *Phyllomedusa burmeisteri* group comprises a compelling example of bisexual polyploids. This group, mostly endemic to the Atlantic forest (AF), is divided in two geographically coherent phylogenetic clades, one composed by the northernmost distributed species (*P. bahiana* and *P. burmeisteri*), and the other including the most southerly distributed taxa (*P. distincta*, *P. iheringii* and the tetraploid species, *P. tetraploidea*; Fig. 5.1) (Brunes *et al*. 2010). Based on cytogenetic data and breeding vocalizations, it has been suggested that *P. tetraploidea* may be originated either by allopolyploidization through hybridization between *P. iheringii* and *P. distincta*, or autopoliploydization from *P. distincta* or *P. iheringii* (Pombal & Haddad 1992; Haddad *et al.* 1994). These authors, based on the undergoing hybridization between *P. tetraploidea* and *P. distincta* with the presence of triploid individuals (Município de Ribeirão Branco, São Paulo state, Brazil; see Fig. 5.1), suggested that *P. tetraploidea* may have resulted from recent autopolyploidy of *P. distincta* as the most plausible hypothesis. Evidences supporting this hypothesis was recently provided by multilocus analysis that placed *P. tetraploidea* and *P. distincta* as sister-taxa, but the inferred Pleistocene origin for the polyploid species (Brunes *et al.* 2010) is not compatible with the previously postulated recent origin. Based on this inferred split time and spatial patterns of genetic variability, these authors also suggested that *P. tetraploidea* could also been originated from an unknown independent lineage that shares a common ancestor with *P. distincta*. Recently, Gruber *et al.* (2013) based on cytogenetic data described a new area of ongoing hybridization close to Ribeirão Branco (Município de Ribeirão Grande, São Paulo state, Brazil; see Fig. 5.1).

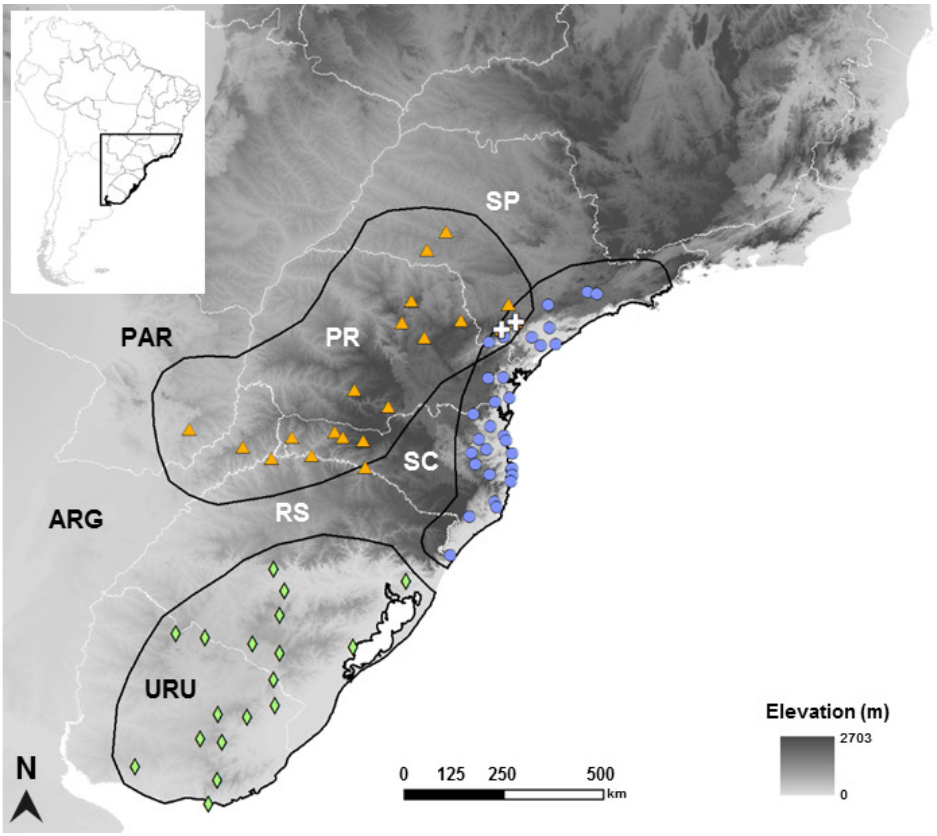


Fig. 5.1 Location of the study area within South America context, geographic range (black lines), and observations of *Phyllomedusa tetraploidea* (orange triangles), *P. distincta* (blue circles), and *P. iheringii* (green rhombus) used in this study. White crosses represent the two areas where *P. tetraploidea* and *P. distincta* are known to hybridize (see text for details). Acronyms in white indicate Brazilian states: SP, São Paulo; PR, Paraná; SC, Santa Catarina; and RS, Rio Grande do Sul. Acronyms in black indicate countries: PAR, Paraguay; ARG, Argentine; and URU, Uruguay.

By exploring both geographic (G)- and ecological (E)-spaces through the application of Ecological Niche-Based Models (ENMs) and multivariate analysis, respectively, here we aimed to perform a niche comparison analysis of the tetraploid (*P. tetraploidea*) and the two closely related diploid species (*P. iheringii* and *P. distincta*) in a fine-scale environmental resolution (climatic, topographical, and habitat variables at 1×1 km grid cell). Specifically, we address the following major question: Are polyploid niche similar or different from those of their diploid relatives regarding range size and environmental requirements? In addition, considering the ongoing hybridization between *P. distincta* and *P. tetraploidea* in two known locations, we expect to identify environmental factors related with the maintenance of their structure and geographical position, and predict the occurrence of other putative diploid-polyploid and diploid-diploid contact zones. Finally, this work aims to provide a framework to the implementation of conservation strategies to preserve geographic areas where these evolutionary processes are ongoing.

5.1.3 Materials and methods

Study area

It has been demonstrated that the success of many ecological niche studies depend on the selection of an appropriate study area (Anderson & Raza, 2010). Thus, here, the study area (308835 pixels of 10km^2) encompasses the know occurrence area of the three endemic species of leaf frogs analysed and covers south-eastern South America, including south-eastern-south Brazil, eastern Paraguay, north-eastern Argentina (Misiones region), and Uruguay. The area is dominated by diverse vegetation types of subtropical Atlantic forest and Pampean forests (Oliveira-Filho *et al.* 2013), with altitude ranging from -15 to 2703 meters (Fig. 5.1).

Observations and ecogeographical variables

We used a total of 20 observations (occurrence points) of *Phyllomedusa tetraploidea*, 32 of *P. distincta*, and 18 of *P. iheringii* (Table D.1). Observations were collected during fieldworks conducted in the south/south-eastern region of the Brazilian Atlantic forest, from December of 2010 to November of 2012, or retrieved from a database of herpetological collections from Argentine, Brazil, Paraguay, and Uruguay (see Acknowledgments). Fieldwork observations were georeferenced with a GPS while observations from collections were based on the city locality (Fig. 5.1). The three species are morphologically distinguishable on the basis of thigh colour pattern (Pombal & Haddad, 1992); however, some misidentification can occur in Ribeirão Branco and Ribeirão Grande, both in São Paulo state (see Fig. 5.1). In these places the diploid species (*P. distincta*) and the tetraploid species (*P. tetraploidea*) hybridize and both specific patterns of thigh coloration, as well as intermediate ones, can be present in the triploids hybrids (Haddad *et al*. 1994). However, both species and their hybrids were karyotypically identified in both hybridization localities by Haddad *et al.* (1994) and Gruber *et al.* (2013). Additionally, to avoid misunderstood related with the use of the occurrence points of these last two species, several *P. distincta* and *P. tetraploidea* observations were molecularly identified through mitochondrial DNA (Brunes *et al.* 2010). The dataset of observations of *P. iheringii* included three molecularly identified observations (Brunes *et al*. 2010) and the extant from two herpetological surveys (Pombal & Haddad 1992; Núñez *et al*. 2004).

We selected a set of 14 bioclimatic, topographical, and land-cover variables at a fine-scale resolution (1×1 km grid cell) with a Pearson correlation lower than 0.75. This set of ecogeographical variables (hereafter EGV's) included: i) five global climate grids from the WorldClim Global Climate Data (Hijmans *et al*. 2005) and two annual measures derivate from this data base: Aridity Index (AI) and Potential Evapo-Transpiration (PET) (Trabucco & Zomer 2009); ii) one topographical grid (USGS 2006) in slope format (extracted from Altitude via 'Slope' function of ARCGIS); and iii) a land-cover grid from the years 2004–2006 (Defourny *et al*. 2009) (Table 5.1). The land-cover grid was converted into six continuous habitat variables by calculating the Euclidean distance ('Euclidean Distance' tool of ARCGIS; e.g. Brito *et al.* 2011) of each grid cell to the closest type of land-cover, for each one of the habitat grids.

Ecological niche-based modelling

To predict the current potential distribution of two diploid and one tetraploid leaf frog species phylogenetically close related in south-eastern South America we generate correlative maps through the maximum entropy algorithm implemented by MaxEnt 3.3.3k (Phillips *et al*. 2006). MaxEnt's method has been widely used to compare species distributions modelling with phylogenetic relationships (Elith *et al*. 2006, 2011, and references therein). Models were built with random training-test percentages (75% of observations for model training, and 25% for model testing) and 10 replicates for each species. We performed a preliminary species-specific tuning (Anderson & Gonzalez, 2011; Radosavljevic & Anderson, 2013), but the results did not differ from those using default parameters suggested by Maxent (data not shown). Quantitative model evaluation was based on a threshold-dependent, minimum training presence (MTP) test omission rates, and a threshold-independent, the Receiver Operating Characteristics Curve (ROC), which identifies the lowest values of average differences between training and test area under the curve (AUC). The use of the ROC statistics to evaluate the accuracy of niche-based models have been recently criticized (see Lobo *et al.* 2008), however, due to the lack of alternative methods in the literature the ROC is still commonly used. The final maps resulted from a binary transformation of species presence and absence based on the MTP value and probabilities below the threshold were transformed to zero. Although choosing a threshold for presence-only analysis has been controversial, the MTP is

considered a conservative approach since it identifies the minimum predicted suitable area allowing for no omission in the training data set (Liu *et al*. 2005; Phillips *et al*. 2006; Pearson *et al*. 2007).

Table 5.1 General information of the environmental variables used to predict the potential niche of *Phyllomedusa distincta*, *P. tetraploidea* and *P. iheringii* with Maxent model at ~1km^2 spatial resolution.

Variable	Mean	Units
Bioclimatic		
BIO 2	Mean Diurnal Range (max temp - min temp)	°C
BIO 4	Temperature Seasonality (standard deviation)	Adimensional
BIO 9	Mean Temperature of Driest Quarter	°C
BIO 13	Precipitation of Wettest Month	mm
BIO 14	Precipitation of Driest Month	mm
AI	Aridity Indexes	mm
PET	Potential Evapo-transpiration	mm
Topographic		
Slope	Slope (derived from altitude)	Degrees
Habitat distance		
DGLC(R)14	Rainfed croplands	m
DGLC(R)20	Mosaic cropland (50-70%) / vegetation (grassland/shrubland/forest) (20-50%)	m
DGLC(R)30	Mosaic vegetation (grassland/shrubland/forest) (50-70%) / cropland (20-50%)	m
DGLC(R)40	Closed to open (>15%) broadleaved evergreen or semi-deciduous forest (>5m)	m
DGLC(R)50	Closed (>40%) broadleaved deciduous forest (>5m)	m
DGLC(R)130	Closed to open (>15%) (broadleaved or needleleaved, evergreen or deciduous) shrubland (<5m)	m

Niche comparisons

Initially, we investigated if there were similar dependences of predicted suitability variables between the niches to identify sympatry probabilities among the aforementioned leaf frogs. For this purpose, the importance of each variable contribution for explaining the potential distribution of each species was estimated by a jackknife test. Variables with smaller differences both in gain and in AUC were the most related to the distribution of species. Thereafter, the visual examination of the profiles of response curves can reveal similarities or differences in the 'optimum' of predicted suitability of variables between species, and therefore can be roughly translated into identical or divergent niche relationships, respectively (e.g. Martínez-Freiría *et al*. 2008).

In a second steep, we measured the level of niche overlap and tested if there

were similarities among the potential distributions of the three species using ENMTools 1.4 (http://enmtools.blogspot.com/). Niche overlap was quantified from Maxent's models generated for each of the three species using Schoener's D (Schoener, 1968), I statistic (Warren *et al*. 2008), both ranging from 0 (no overlap) to 1 (complete overlap). In both cases, the level of overlap was calculated by taking differences between species in suitability scores at each grid cell, after suitabilities were standardized so that they sum to 1 over the geographical space being measured. Subsequently, the null hypothesis that species exhibits identical potential distributions was tested with a pairwise 'Identity test' through 50 randomized pseudoreplicates. By comparing the observed values of D and I to the null distribution obtained using the hypothesis test, it was possible to determine whether pairwise models produced statistically significantly different results (Warren *et al*. 2008, 2010).

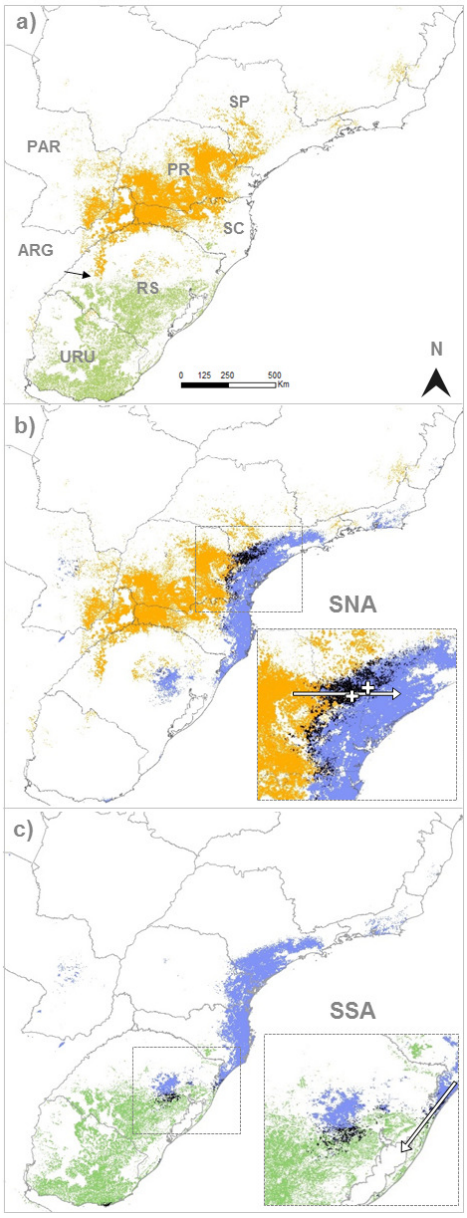
Finally, differences and similarities among leaf frog's niches were explored via multivariate analysis. We performed a Spatial Principal Component Analysis (SPCA) using ARCGIS version 10.1 (ESRI 2012). Analyses were performed with standardized EGV's most related with the potential presence of the three species (see the results in Fig. D.1). To avoid spurious representation (i.e. including areas where none of the three species occurs), we reduced the study area (hereafter adjusted study area) based on the potential distributions generated by the Maxent models. The two first components and the potential distributions were converted into point data shapefiles using the centroid of each grid cell. Then, we randomly selected 1% of the total points using the Hawth's Analysis Tools for ArcGIS (Beyer 2004). Finally, we extracted the scores of the components that explain the highest variance of the adjusted study area, as well as the potential distributions and occurrence information of the three species, and plot them.

Putative sympatric areas

The geographic identification of putative sympatric areas among all species was determined by the overlap of threshold models predicted by Maxent, using the 'Combine' function of ARCGIS. We verified if these sympatric areas overlap to classical transitional areas which favour the occurrence of both species involved through smooth environmental shifts. For that, we draw two transects (300km) based

on the size of the putative sympatry areas to characterize both species-specific environment and the area shared by the reloaded species. We restricted analyses to the EGV's most related with the potential presence of the three species (see results in Fig. D.1) and evaluated each profile and range of variation over 60 points sampled every 5 to 5km. In addition, the location of transects were contrasted with the maps of the components that explain the highest environmental variance revealed by the SPCA.

5.1.4 Results



Predicted species distribution

The ROC plots presented high mean AUC's test (0.905-0.977) and low values of both standard deviations (0.009-0.036) and differences between training and test AUC (0.013 to 0.068) for the three species models. The average omission rates based on the MTP test varied from 0.1 to 0.33 and the MTP based only in the observations used to produce the model predictions were: 0.1 to *P. distincta*, 0.18 to *P. tetraploidea*, and 0.21 to *P. iheringii* (Table 5.2). The Maximum Entropy models predicted areas with high environmental suitability compatible with the current distribution of the three species (Fig. 5.2).

Fig. 5.2 Combination of current environmental suitable areas between: a) Phyllomedusa tetraploidea (orange) and P. iheringii (green); b) P. tetraploidea and P. distincta (blue); and c) P. distincta and P. iheringii predicted by Maxent model in south-eastern South America. Black arrow indicates the overlap area between P. tetraploidea and P. iheringii. Amplified areas shows the range of the two potential sympatry areas between species and white arrows indicate the position/direction of the analysed transects in the Fig. 5.5. White crosses represent the two places where natural hybridization was described between P. tetraploidea and P. distincta; RB, Ribeirão Branco and RG, Ribeirão Grande (see text for details). SNA, Sympatric Northern area; SSA, Sympatric Southern area. Acronyms indicate Brazilian states: SP, São Paulo; PR, Paraná; SC, Santa Catarina; and RS, Rio Grande do Sul; and countries: PAR, Paraguay; ARG, Argentine; and URU, Uruguay.

The three models showed extremely small overprediction areas. In general, the size of the potential distribution area projected for *P. tetraploidea* and *P. iheringii* were similar (~12%) and for *P. distincta* was significantly lower (~4%) regarding the size of the study area (Table 5.2).

Table 5.2 Model evaluation, threshold and projection area for *Phyllomedusa distincta*, *P. tetraploidea*, and *P. iheringii* carried out with 10 replicated runs using climatic, topographic and habitat distance under Maxent model. See text for abbreviations details. Regularization parameter value were 1.0 (default) for all species.

Maxent's overview	*P. distincta*	*P. tetraploidea*	*P. iheringii*
Number of total samples	32	20	18
Number of model samples (training - test)	24-8	15-4	14-4
Average test AUC for the replicate (SD)	0.977 (0.009)	0.905 (0.036)	0.922 (0.021)
AUC differences (training - test)	0.013	0.068	0.042
MTP test omission rates	0.1	0.33	0.25
MTP of occurrence points	0.1	0.182	0.215
Projection area (~10km^2) (%)	13606 (4.41)	38021 (12.31)	36770 (11.91)

Niche comparisons

The jackknife test identified the variables most related with the potential presence of the leaf frogs in the study area (Fig. D.1). The distribution of leaf frogs was influenced by common EGVs, such as max-min temperatures and seasonality, precipitation, aridity indexes, and evapo-transpiration (BIO 2, 4, 13 e 14, AI and PET). The distribution of *P. tetraploidea* was related to temperature seasonality, precipitation, aridity indexes, and one habitat type; *P. distincta* was related to max-min temperatures and seasonality, potential evapo-transpiration, and aridity indexes; and *P. iheringii* was related to temperature seasonality, precipitation, and evapo-transpiration. The comparison of the response curves profiles for the EGV's related to the distribution of two or more species allowed to explore niche characteristics shared by the studied species (Fig. 5.3). Overall, the probability of presence of *P. distincta* was highly related with low values of thermal amplitude, on the contrary of *P. tetraploidea*, while in turn, *P. iheringii* occurred in areas with intermediate values. The species *P. iheringii* occurs in areas with prominent seasonality in terms of temperature. The response curve profiles also revealed bioclimatic relationship between pairs of leaf frogs that may be translated in probable sympatric conditions: 1) *P. tetraploidea* and *P. distincta* occurs in areas with similar temperature seasonality (Fig. 5.3b), high levels of rainfall during the wettest month (Fig. 5.3c), and with a strong tendency to fixate their distribution in humid areas (Fig. 5.3f); and 2) *P. distincta* and *P. iheringii* occurred in areas with similar mean diurnal range (Fig. 5.3a),

rainfall levels during the driest month (Fig. 3d) and potential evapo-transpiration (Fig. 5.3e).

Metrics of niche overlap presented lower values for the niche occupied by *P. iheringii* when compared with both *P. tetraploidea* and *P. distincta*. All values were significantly lower than expected under the null hypothesis of niche identity (Table 5.3). The first and second axis of the SPCA explained a total of 75% of the environmental variation of the study area (Table 5.4). The highest variable loadings in PC1 were PET, BIO 2, and BIO 14; these factors represent areas with low thermal amplitude, high potential evapo-transpiration, and also areas with low precipitation during the driest season. The PC2 represents regions with well-defined seasons and humid areas with elevated precipitation (BIO 4, 13 and AI). In general, the first two axes presented similar clines and differentiated three ecological spaces for the leaf frogs in south-eastern South America (Fig. D.2). A scatterplot representing SPCA scores of localities with species observations and predicted potential distribution (Fig. 5.4), showed a tendency for niche divergence for the three species and revealed close relationships between the niche of *P. tetraploidea* and *P. distincta*. In addition, the two described hybrid zones between these last two species showed a central position in the overlapped area.

Table 5.3 Tests of niche equivalency. Similarity scores for ENMs of *Phyllomedusa distincta*, *P. tetraploidea* and *P. iheringii* (Fig. 5.2) implemented in ENMtools 1.4.3. Niche overlap measures: Schoener´s D below of the diagonal and Hellinger´s I above. The two metrics range from 0 (discordant ENMs) to 1 (identical ENMs). Parentheses indicate mean and standard deviation. All values were significant (*t*-test: $P < 0.01$) via randomization tests (see text).

Species	*P. distincta*	*P. tetraploidea*	*P. iheringii*
Phyllomedusa distincta	1	0.47 (0.87 ±0.031)	0.32 (0.88 ±0.041)
Phyllomedusa tetraploidea	0.23 (0.64 ±0.047)	1	0.28 (0.89 ±0.043)
Phyllomedusa iheringii	0.13 (0.64 ±0.064)	0.11 (0.66 ±0.064)	1

Table 5.4 Percentage of total variance, top loadings and environmental interpretation explained by the first two principal components extracted according to the SPCA analysis of the fitted study area occupied by *Phyllomedusa tetraploidea*, *P. iheringii*, and *P. distincta* (see text for details).

	PC1	PC2
% variance explained	41.85	32.47
Top variable loadings	PET (0.557) BIO 2 (0.487) BIO 14 (-0.441)	BIO 4 (-0.620) BIO 13 (0.640) AI (0.413)
Interpretation	High temperatures variation and low precipitation in dry season	High temperature stability and precipitation in wet season

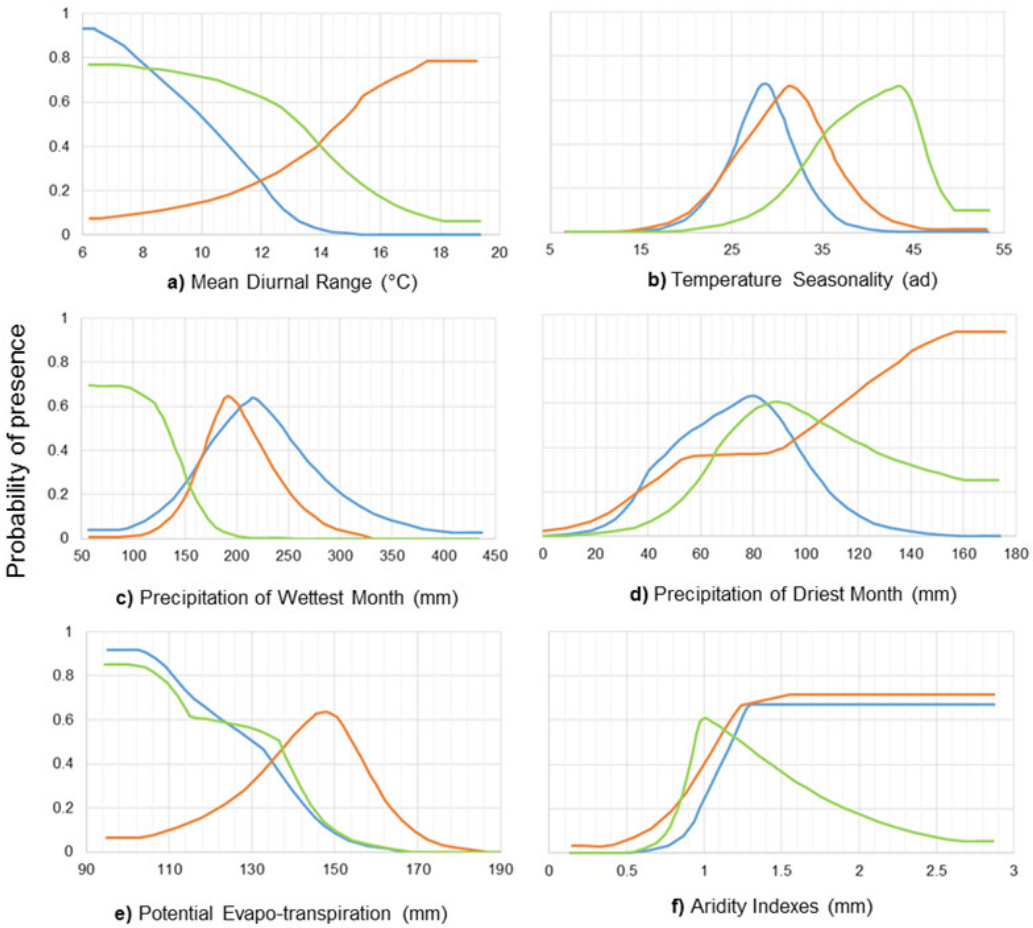


Fig. 5.3 Comparison of response curves (a-f) for the environmental variables most related with the potential presence of *Phyllomedusa distincta* (blue), *Phyllomedusa tetraploidea* (orange) and *Phyllomedusa iheringii* (green) extracted from Maxent models performed in southeastern South America region.

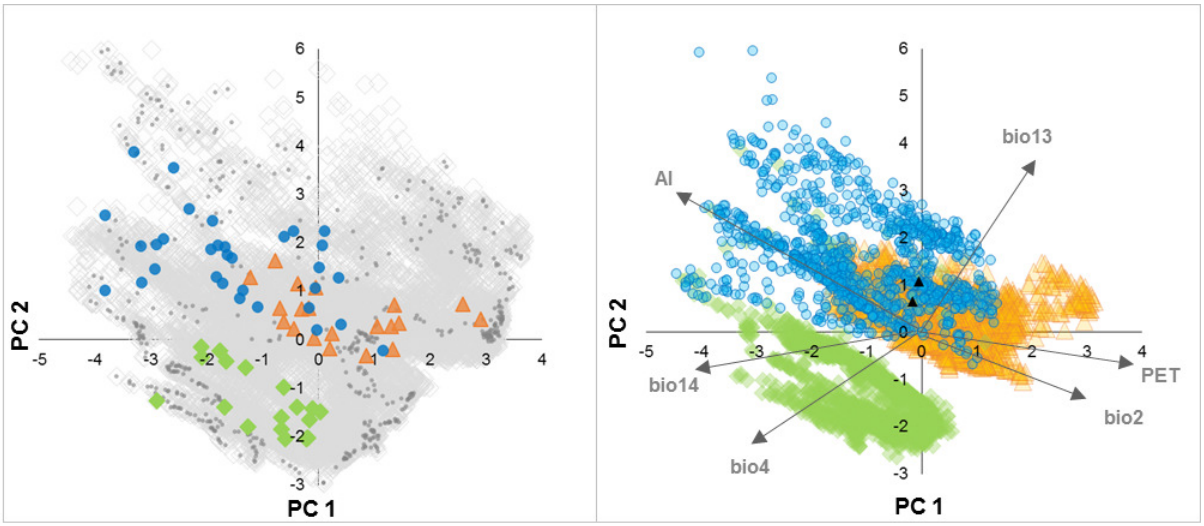


Fig. 5.4 Adjusted study area with the occurrence localities (a) and potential distribution (b) of *Phyllomedusa tetraploidea* (orange), *P. distincta* (blue), and *P. iheringii* (green) in a graphic representation of a Spatial Principal Component Analysis (SPCA). Small dark gray points in figure (a) represent the geographical limits of the adjusted study area. The SPCA was based on the six ecogeographical variables most related with the potential presence of the three species (see Fig. E.1). Black triangles locate the two described natural hybrid zones between *Phyllomedusa tetraploidea* and *Phyllomedusa distincta* (SNA) in figure (b) (see Fig. 5.2 for details).

Putative sympatric areas

Putative sympatric areas between leaf frogs were identified in Brazilian territory between: i) *P. tetraploidea* and *P. distincta* (hereafter Sympatric Northern Area, SNA) around the two known localities where these species hybridize (Ribeirão Branco and Ribeirão Grande; see Introduction for details), mainly located in south-eastern of São Paulo state; and ii) *P. distincta* and *P. iheringii* (hereafter Sympatry Southern Area, SSA) in the southern and northern limits of distribution of both species, respectively, located among Santa Catarina and Rio Grande do Sul states and in an fragmented area in central Rio Grande do Sul (Fig. 5.2b and c). The ENM's found little evidences for the putative sympatry between *P. tetraploidea* and *P. iheringii* (Fig. 5.2a), corresponding only to 194 grids cells (1×1km squares). No putative sympatry was detected when dealing with three species together (data not shown).

The inspection of the environmental profile of the six most important EGV'S along both transects drawn in the two putative sympatry areas presented contrasting results (Fig. 5.5). The probabilities of presence were higher in the SNA (~0.6) and showed a balanced pattern which is represented by an exclusive tendency for each species to occur at the edges and both at the middle. The same pattern was not so clear in the SSA (~0.3). Regarding the spatial variation in the six EGV'S, there was a gradual tendency of increase or decrease along the SNA transect. On the contrary, the environmental variations along the SSA transect were sharper, excepting the precipitation of the wettest month which was constant.

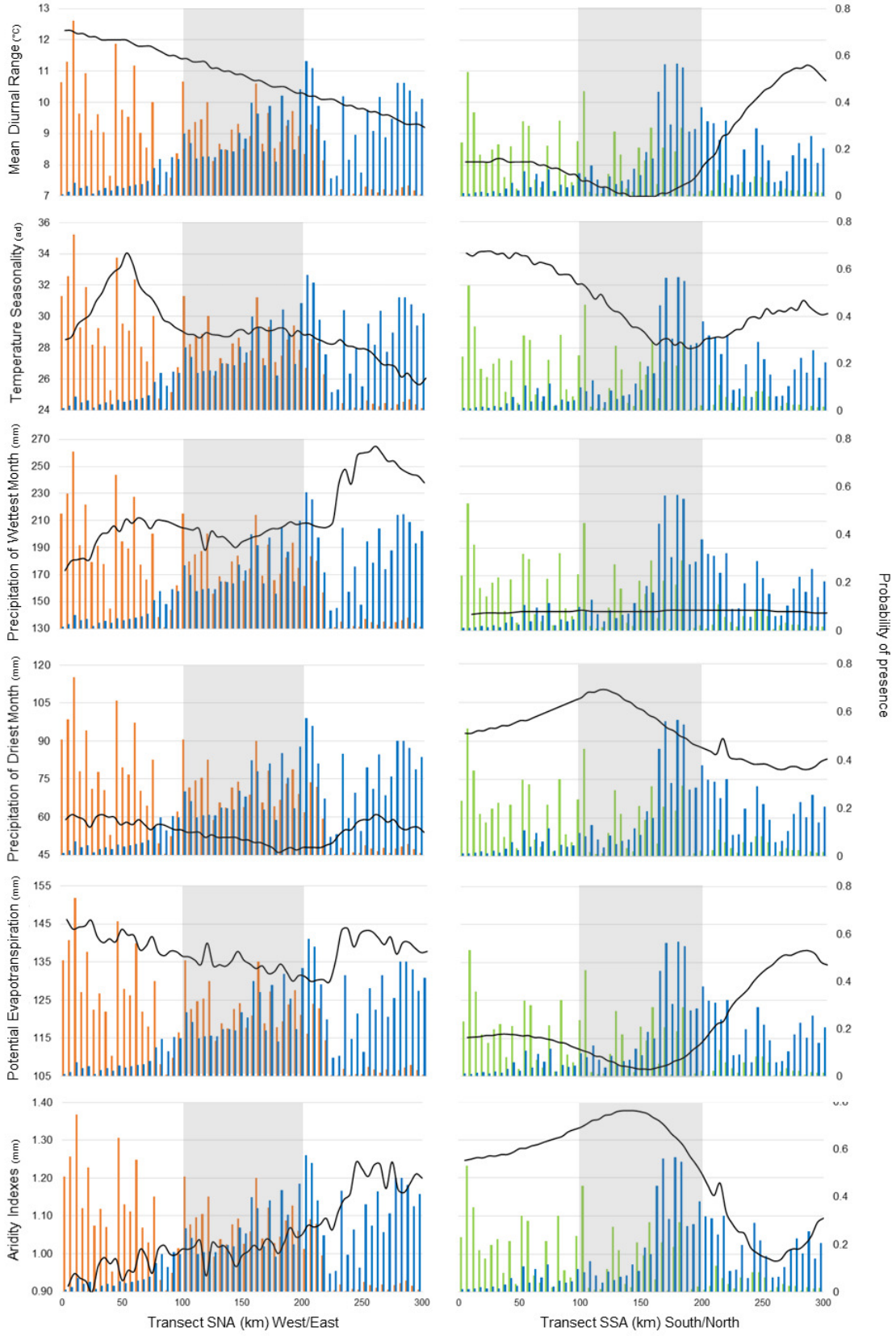


Fig. 5.5 Environmental characterization of the putative sympatry areas between: *Phyllomedusa tetraploidea* and *P. distincta* (SNA); and *P. iheringii* and *P.distincta* (SSA) in the south-eastern/south Brazilian Atlantic forest. Both transects were divided in three areas of 100 km based on a combination of the potential distribution of both species predicted by Maxent (See Figs. 5.2b-c and 5.3). Black lines represents the dispersion of each variable value sampled every 5 km. Grey areas represent the range of both putative sympatry areas.

5.1.5 Discussion

Predicted species distribution

The ecological niche-based modelling of two diploid and one tetraploid leaf frogs from south-eastern South America predicted areas with high environmental suitability, compatible with their current known species distributions (Figs. 5.1 and 5.2). This high level of coincidence is likely explained by the use of a comprehensive set of ecogeographical variables (e.g. climatic, topographical, and land cover). In fact, the performance of fine-scaled ecological models are showing an accurate definition to predict suitable habitats for different organisms and environmental conditions around the globe (see Cord & Rödder 2011; Brito *et al.* 2011). The size of the potential distribution area predicted for *P. distincta* (~4% of the study area) was lower than that for *P. tetraploidea* and *P. iheringii* (~12%) and mainly restricted to the Atlantic coast, region covered by the well-defined Atlantic rainforest habitat. This result can roughly suggest that *P. distincta* is ecologically restricted. The projected area for *P. tetraploidea* was in agreement with the assumptions that this is the unique species from *P. burmeisteri* group with exclusive inland distribution (Pombal & Haddad, 1992).

Ecogeographical requirements

The ecological models identified the set of variables most related with the potential presence of the leaf frogs in south-eastern South America. Overall, the distribution of leaf frogs are more related with bioclimatic factors than with habitat characteristics. Within bioclimatic factors, variables with well-known high physiological importance for frogs, such as temperature variation and distribution of precipitation, were the most related with the distribution of the three species of leaf frogs that we studied. Indeed, although species from the genus *Phyllomedusa* are commonly associated to forested habitats, mainly due to its reproductive mode based on a leaf nest pending over water (Mode 24 of Haddad & Prado 2005), field observations suggest that they can cope well with moderate anthropogenic disturbance, including agricultural areas. For instance, *P. distincta* was found reproducing in ponds surrounded by exotic eucalyptus tree and *P. iheringii* in soybean crops (T. Brunes and J. Alexandrino personal observations). Both types of monocultures are in rapid expansion in the Atlantic forest being considered as one of the major threats for biodiversity of this

biome (Ribeiro *et al*. 2011). Although these leaf frogs can reproduce in this type of disturbed areas, the decrease of connectivity between populations, and the additional edge effect, can act negatively on the genetic variability by reducing gene flow among populations.

Are there differences between the niches of diploid and polyploid species?

In nature, the distribution of polyploids has been associated with harsher habitats and climatically unstable regions (Stebbins 1950; Soltis & Soltis 1999; Otto *et al*. 2007). Following this hypothesis, we expect to find closer relationships between niches of diploid-diploid species than niches between diploid-tetraploid species. Our results are not fully congruent with this hypothesis (Fig. 5.3). On one hand, three of the six environmental factors suggest that *P. tetraploidea* and *P. distincta* share a preference for habitats with more stable annual temperatures, higher levels of precipitation during the wettest month, and slightly more humid conditions (aridity value), when compared to the 'optimum' values for *P. iheringii*. These results are congruent with the fact that there are areas where *P. tetraploidea* and *P. distincta* hybridize producing triploid individuals (see Fig. 5.1; Haddad *et al.* 1994; Gruber *et al.* 2013). On the other hand, the remaining three variables suggest that *P. distincta* and *P. iheringii* can co-occur in areas with medium levels of high precipitation in the driest months and potential evapo-transpiration, besides of areas with smaller differences in the mean diurnal ranges, when compared to *P. tetraploidea*. Although the probability of occurrence along the aridity index gradient was relatively similar for the three species, *P. distincta* and *P. tetraploidea* occur preferentially in humid areas, while *P. iheringii* occurs in slightly drier areas. Overall, for all diploid-diploid and diploid-polyploid niche comparisons, our results were congruent in showing that the more divergent niches are between the diploid *P. iheringii* and the polyploid *P. tetraploidea*. However, based on known field records either *P. distincta* and *P. iheringii* or the three species together do not co-occur (Pombal & Haddad 1992; Brunes *et al*. 2010). Both multivariate analysis and niche overlap measurements support a higher niche similarity between the diploid species, *P. distincta* and the polyploid one, *P. tetraploidea* when compared with *P. iheringii* (Table 5.3; Fig. 5.4). While this result runs parallel to the sister-taxa relationship between *P. tetraploidea* and *P. distincta* as inferred by multilocus phylogenetic analyses (Brunes *et al*. 2010), the influence of

phylogeny and ploidy on species distribution in animals is still waiting for future detailed studies (see below).

Regarding the hypothesis that polyploidy species occur in harsher environments and have broader distribution ranges than their diploid counterparts, our ecological models and multivariate analyses suggest that the two diploid species occur in areas with both lower thermal amplitude and evapo-transpiration compared to tetraploid species (Table 5.3 and Fig. 5.4). Despite the lack of experimental studies about the impact of thermal amplitude in this group, our results suggest that *P. tetraploidea* is adapted to higher thermal amplitudes than diploid species, exhibiting higher tolerance to climate oscillations regime (see Folguera *et al*., 2009). Similarly, higher potential evapo-transpiration could be related with high temperatures in the absence of compensation by precipitation. These results suggest that *P. tetraploidea* occupies areas with more severe habitat conditions. Although species from the genus *Phyllomedusa* are considered "waterproof frog" by presenting eco-physiological adaptations linked to desiccation resistance (see Shoemaker *et al*. 1972; Faivovich *et al*. 2010), *P. tetraploidea* seems to benefit from its duplicated genome for adaptation to harsher habitats. This hypothesis is not confirmed by the analyses of Mable *et al*. (2011) in several bisexually reproducing amphibians and fishes. These authors used the Köppen classification of climatic zones to contrast diploid-polyploid distributions following the hypothesis that polyploids occur in more extreme environments than their close diploid relatives. However, the lack of fine-scale resolution of the Köppen classification may have hidden such differences. Indeed, Otto *et al*. (2007), using a similar approach used herein, found the same general evidences of those reported for *P. tetraploidea*. Notwithstanding, it is also possible that occurrence in harsher habitats by the tetraploid species reflects their phylogeny, instead of adaption due to polyploidy (e.g. Martin & Husband, 2009). Although our results do not bear this relationship, future studies are needed to elucidate this question, including the use of integrative methods of phylogenetics and climatic niche modelling (e.g. Heibl & Calenge, 2013).

Are the potential sympatric areas environmentally realistic?

Ecological models predicted two putative sympatric areas between leaf frogs. One area involves diploid-polyploid species (*P. distincta* and *P. tetraploidea*) in a narrow

band along a 200 km long latitudinal axis (SNA), and the other area involves diploid-diploid species (*P. distincta* and *P. iheringii*) in a small restricted area (Fig. 5.2b-c). These results mirror the profiles of response curves in revealing niche similarity between diploid-polyploid (SNA) and diploid-diploid (SSA). Interestingly, both areas roughly coincide with transition areas of vegetation types (see Oliveira-Filho *et al.* 2013). Despite of this, a visual inspection of graphs representing the spatial climatic variables across the putative sympatric areas (Fig. 5.5) and their respective positions in SPCA analysis (Fig. D.2), showed that SNA transect is located in an area with smooth climatic transition, while the SSA exhibits abrupt variations. This last result suggests an extremely reduced chance of hybridization between diploid species'. Nevertheless, future extensive field works in the SSA region are necessary to confirm the absence of hybridization.

In the case of *P. distincta* and *P. tetraploidea*, our results corroborate previously documented localization of the natural hybrid zone, where these species apparently hybridize in the absence of pre-zygotic barriers (indistinguishable advertisement calls). Temporal and phenotypic assortative mating seems also to be absent through field observations (Haddad *et al*. 1994) and from the direction of mitochondrial DNA in crosses cytogenetically characterized (C.F.B. Haddad and S. Kasahara, unpublished data). Although triploid individuals mate with both parental (diploid and tetraploid) and triploids, field and laboratory observations showed that triploids have substantially lower number of eggs per clutch compared to parental species (Haddad *et al*. 1994) and have degenerate chromosomes in initial meiotic stages (Gruber *et al*. 2013), both indicative of low fertility. While these results suggest that the diploid-polyploid hybrid zone fit a tension zone model with strong selection acting against hybrids (Barton & Hewitt 1981 1985), the occurrence of triploids in higher frequency in the area of overlap (Haddad *et al*. 1994), that is ecologically transitional, could also suggest that the environment also plays an important role for the current position of this hybrid zone.

Conclusions

This study represents the first work that combines a comprehensive set of climatic, topographical, and habitat variables to explore both G- and E-spaces of a diploid-polyploid amphibian group. Our findings on the ecological niches provided

quantitative evidences that support the widely debated hypothesis for the tendency towards polyploid species occur in more severe habitats than the diploid sister species. In particular our fine-scale GIS-based analyses were useful to provide new evidences on polyploidy origin and in the identification of factors affecting both location and maintenance of natural hybrid zones in this leaf frogs group. Considering that approximately 10 cases of polyploidy (see reviews in Bogart 1980; Mable *et al.* 2011) in several anuran species have already been described for south-east America, this approach could be seen as a starting point for future comparative studies of the role of biogeography in polyploid origin, evolution, and distribution. In a more broad sense, regarding the role of evolutionary processes such as polyploidy and hybridization in originating and sustaining diversity, as well as in generating taxonomically complex groups, this approach may also be particularly useful to define priority areas for conservation of these processes instead of the traditional species-based conservation programs (Ennos *et al*. 2005) at both macro and micro-scale perspectives.

5.1.6 Acknowledgements

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5.1.7 References

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Capítulo 6

Discussão Geral

6.1 Filogenia: árvores de genes *versus* espécies

O primeiro grande objetivo desta tese foi o uso de diferentes marcadores moleculares (mitocondriais e nucleares) para avaliar, numa primeira fase, a correspondência entre os dados moleculares e a divisão do grupo *P. burmeisteri* em cinco espécies definidas com base em carateres morfológicos e, numa segunda fase, estimar as suas relações filogenéticas. Com efeito, até à realização deste estudo, os poucos trabalhos realizados sobre o grupo *P. burmeisteri* enquadravam-se em objetivos mais amplos, como por exemplo, o estudo da filogenia da família Phyllomedusinae (Faivovich *et al*. 2010), em que foram usados poucos exemplares (1-3) de cada espécie. Assim, neste primeiro estudo (Secção 2.1), a caracterização genética de vários exemplares de cada espécie do grupo através do uso de um gene mitocondrial e dois genes nucleares de cópia única, recorrendo a métodos recentes de análises multilocus (árvore de espécies), permitiu recuperar cada espécie, previamente definida morfologicamente, como um grupo monofilético. Este trabalho sugeriu ainda que estas espécies estão divididas em dois grupos principais: 1) um formado pelas espécies distribuídas mais ao norte (*P. bahiana* e *P. burmeisteri*) e um outro, 2) formado pelas espécies distribuídas mais ao sul (*P. distincta*, *P. tetraploidea* e *P. iheringii*). Estes resultados mostraram ser assim discordantes relativamente à inferência de *P. bahiana* como espécie basal do grupo (Faivovich *et al*. 2010), mas congruentes com a maior semelhança entre as vocalizações descritas por Pombal & Haddad (1992) entre as espécies de distribuição mais ao norte e sul, respetivamente. Além disso, os resultados mostraram que *P. distincta* e *P. tetraploidea* são espécie-irmãs, o que constitui uma evidência adicional para a hipótese da origem da forma tetraploide por um processo de autopoliploidização a partir de *P. distincta* previamente sugerida por Pombal & Haddad (1992) (ver discussão detalhada na Secção 2.1)

Outro dos aspetos mais importantes deste estudo foi a utilização de métodos de análise multilocus recentes com base no coalescente, contribuindo para o debate sobre as limitações dos estudos filogenéticos baseados quer na análise de genes mitocondriais apenas, quer no uso de métodos tradicionais concebidos para a análise de um único gene (concatenação) para inferências multilocus (Kubatko & Degnan 2007; Degnan & Rosenberg 2009; Heled & Drummond 2010). Este aspeto reveste-se da maior importância para vários taxa filogeneticamente aparentados ou

grupos de espécies crípticas, em que processos como rápidas radiações adaptativas ou hibridações resultam frequentemente em discordâncias entre marcadores citoplasmáticos e nucleares, podendo conduzir a fortes inconsistências na inferência das relações filogenéticas (Belfiore *et al.* 2008; Themudo *et al.* 2009). A este respeito, refira-se em particular os estudos em regiões de elevada biodiversidade, como por exemplo os Neotrópicos, em que o uso apenas de marcadores citoplasmáticos continua a ser uma prática comum (Ramos *et al.* 2009; Trinca *et al.* 2012; Maldonado-Coelho *et al.* 2013). Por último, refira-se ainda a importância de se ter usado um número de exemplares relativamente elevado e representativo da área de distribuição das espécies do grupo *P. burmeisteri* (4-23 por espécie) ao longo da Mata Atlântica (MA), fato que permitiu observar elevados a moderados níveis de estruturação genética intraespecífica, que se traduz na existência de várias linhagens com trajetórias evolutivas independentes.

6.2 Padrões e processos de diversificação biológica na Mata Atlântica

Os processos que estão na base dos elevados níveis de biodiversidade encontrados na MA desde sempre intrigaram vários investigadores. Estudos moleculares recentes confirmaram as previsões teóricas que a história biogeográfica de muitas espécies foram afetadas por processos cíclicos de isolamento e persistência de organismos em refúgios florestais em consequência das flutuações climáticas do Quaternário – Hipótese dos refúgios do Pleistoceno (*sensu* Haffer, 1969; ver Secção 1.2.3). Outros estudos, no entanto, sugeriram que a diversidade biológica não pode ser explicada unicamente pela ocorrência de flutuações climáticas do Quaternário, mas que também fatores climáticos e geológicos, como os grandes rios, cadeias montanhosas e eventos tectônicos ocorridos durante o Terciário contribuíram para os padrões atuais de diversidade genética e de diversificação de muitos organismos da BAF (Pellegrino *et al.* 2005; Grazziotin *et al.* 2006). Mais recentemente, um estudo baseado em modelos paleoclimáticos da MA (Carnaval & Moritz 2008) sugeriu a ocorrência de variação espacial na persistência das florestas ao longo do Pleistoceno. De acordo com os modelos obtidos, estes autores sugeriram a existência de uma área de estabilidade de floresta relativamente grande no corredor central (Bahia) e outra mais pequenas (Pernambuco) ao longo da costa brasileira,

aproximadamente concordantes com os centros atuais de endemismo (Lynch 1979; Costa *et al.* 2000) e padrões de diversidade genética (mtDNA) de três espécies de sapo-folha endémicos da MA (Carnaval *et al.* 2009).

Considerando a ampla distribuição geográfica das espécies de *Phyllomedusa* do grupo *burmeisteri* e também a elevada heterogeneidade dos habitats de ocorrência (ver Secção 1.3.2), procurou-se, como primeiro objetivo, identificar quais os principais mecanismos de diversificação e os fatores responsáveis pelos atuais padrões biogeográficos. Para isso recorremos ao uso de um gene mitocondrial e duas genealogias nucleares para analisar vários exemplares representativos da área de distribuição de cada uma das espécies. Os resultados obtidos sugeriram que tanto os períodos Terciário como o Quaternário foram importantes para a diversificação de espécies do grupo *P. burmeisteri.* De acordo com as estimativas temporais (tMRCA), obtidas com base na árvore de espécies multilocus, a divergência entre os dois principais grupos de espécies (norte e sul, ver Secção 6.1) deste complexo ocorreu durante o Miocénico (~ 5 milhões de anos atrás), enquanto dentro destes grupos a diversificação ocorreu desde o Terciário tardio (Plioceno) e, essencialmente, durante todo o Pleistoceno. Estas estimativas temporais coincidiram largamente com as obtidas para outros organismos amplamente distribuídos na BAF (Grazziotin *et al.* 2006; Fitzpatrick *et al.* 2009; Thomé *et al.* 2010; Amaro *et al.* 2012).

Outro dos resultados mais importantes deste estudo diz respeito aos padrões espaciais de diversificação, tendo-se verificado que na região sul do estado de São Paulo, onde ocorre a separação entre o grupo norte (*P. burmeisteri* e *P. bahiana*) do grupo sul (*P. tetraploidea*, *P. distincta*, e *P. iheringii*) do complexo *P. burmeisteri*, também ocorre a separação de grandes clados de outos organismos da MA, incluindo aves (Cabanne *et al.* 2008), répteis (Grazziotin *et al.* 2006) e, principalmente anfíbios (Carnaval *et al.* 2009; Thomé *et al.* 2010; Amaro *et al.* 2012). Além disso, este padrão espacial é também coincidente com os padrões de endemismo para anfíbios e mamíferos da BAF (Lynch 1979; Costa *et al.* 2000) e ainda com a área de estabilidade de floresta mais a sul previsto por modelos paleoclimáticos da BAF (Carnaval & Moritz, 2008). Esta coincidência ao nível dos padrões espaciais de diversidade genética e de biodiversidade sugere que a diversificação biológica pode ter resultado da atuação de processos comuns. Embora este estudo não tenha permitido identificar tais processos, com base nos conhecimentos atuais sobre a ocorrência de eventos geomorfológicos durante o

Terciário até aos dias de hoje, como a modificação de partes da costa leste brasileira, devido a fenômenos neotectônicos mais antigos e também recentes, assim como o complexo sistema de rios da MA, permite avançar algumas hipóteses explicativas (ver Ribeiro 2006). Por exemplo, a zona onde se verifica a separação entre os dois principais grupos do complexo de *P. burmeisteri*, no sul de São Paulo, é coincidente com os movimentos espaciais do lineamento de Guapiara, enquanto a zona de cisalhamento de Cubatão (Fig. 6.1) parece estar associada à divisão das duas linhagens observadas em *P. distincta* (Saadi *et al.* 2002).

Enquanto os resultados deste primeiro estudo conduziram à elaboração das primeiras hipóteses sobre os padrões gerais de diversificação quer ao nível espacial quer ao nível temporal das espécies do grupo *P. burmeisteri*, o estudo detalhado dos padrões filogeográficos de *P. burmeisteri*, *P. bahiana*, e *P. distincta* permitiram aprofundar os conhecimentos sobre a história biogeográfica e os principais fatores responsáveis pelos padrões atuais de estrutura genética. No caso de *P. burmeisteri* e *P. bahiana*, os resultados obtidos através da análise de sequências de mtDNA e três genealogias nucleares, não corroboraram a existência de uma extensa área de hibridação previamente sugerida por Pombal e Haddad (1992). Com efeito, o número de indivíduos miscigenados ao nível nuclear ou com evidências de introgressão ao nível do mtDNA são relativamente poucos e restritos a algumas localidades do sul da Bahia e no Espírito Santo (ES). Interessante é a ocorrência de haplótipos de mtDNA de *P. bahiana* e *P. burmeisteri* numa mesma localidade (Aracruz) próxima do rio Doce (Fig. 6.1). Os indivíduos portadores de haplótipos de *P. bahiana* foram classificados como P*. burmeisteri* com base em marcadores nucleares, sugerindo assim que a presença de ambos os haplótipos nesta região pode ser um remanescente de uma distribuição mais ampla de *P. bahiana* no passado.

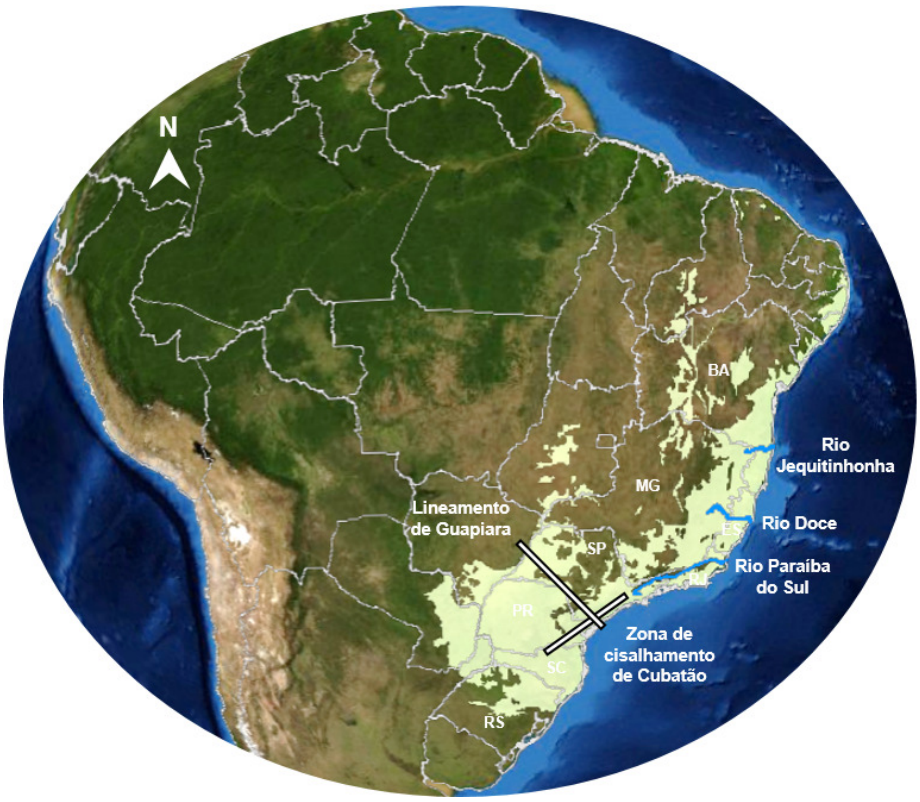

Fig. 6.1 Mapa parcial da América do Sul representando a cobertura original do bioma Mata Atlântica, os principais rios da bacia do Atlântico Leste e as falhas neotectônicas que podem ter influenciado o processo de diversificação das espécies do grupo *P. burmeisteri*. Escala: 1:50.000.000. Fonte: SOS Mata Atlântica.

Assim, estes resultados parecem refletir a combinação de episódios distintos de contato secundário entre *P. bahiana* e *P. burmeisteri*: um evento de hibridização mais antigo na região de ES, e um contato secundário mais recente no sul da Bahia. A persistência de mtDNA de uma outra linhagem/espécie como resultado de uma hibridação passada é um fenómeno muito frequente em anfíbios (Babik *et al.* 2003; Sequeira *et al.* 2005, 2011) podendo em alguns casos estar associado ao movimento de zonas híbridas. Um outro resultado que parece dar algum suporte à existência de hibridações passadas nesta região resultou da análise de um conjunto de 11 microssatélites através da aplicação de métodos Bayesianos de agrupamentos multilocus e análises multivariadas. Os resultados destas análises mostraram que a população de Aracruz corresponde a um dos quatro grupos populacionais geneticamente diferenciados em *P. burmeisteri*. Com efeito, é possível que em consequência das oscilações climáticas do Pleistoceno, com a concomitante alteração do habitat, *P. bahiana* se tenha retraído mais para norte, enquanto *P. burmeisteri* expandia a sua área de distribuição no mesmo sentido. Enquanto esta hipótese carece de uma análise mais aprofundada, há evidencias que sugerem a influência quer do rio Doce quer do rio Jequitinhonha (Fig. 6.1) na atual história

biogeográfica destas espécies. Efetivamente, o sistema do rio Doce tem sido apontada como uma importante barreira para outros organismos da BAF (Pellegrino *et al.* 2005; Lara-Ruiz *et al.* 2008; Thomé *et al.* 2010; Ribeiro *et al.* 2011a; Tonini *et al.* 2013).

Outro dos resultados importantes deste estudo foi a deteção de uma linhagem mitocondrial altamente divergente de *P. burmeisteri* geograficamente restrita ao estado do Rio de Janeiro (BUR-RJ; *p*-não corrigida=7%). De acordo com a estimativa de tempo obtida a partir da árvore de espécies multilocus esta linhagem ter-se-á separado há aproximadamente 1 milhão de anos atrás. Este grupo ocorre numa região topográfica e fitogeograficamente complexa que abrange o vale do Paraíba do Sul (Fig. 6.2) e várias cadeias de montanhas pertencentes à Serra do Mar, que se caracteriza por apresentar acentuados gradientes altitudinais (>1000 m) e fortes transições climáticas e fitofisionómicas (IBGE, 2012). Estas características, juntamente com evidências palinológicas que sugerem alterações das condições do habitat devido às oscilações climáticas do Quaternário, provavelmente criaram condições para a diversificação e persistência de populações. Os resultados aqui obtidos são importantes para o amplo debate sobre refúgios do Pleistoceno na BAF (Carnaval & Moritz, 2009). De facto, a ocorrência desta unidade evolutiva de *P. burmeisteri* aliado aos níveis elevados de espécies endémicas (Rocha *et al.* 2004) e linhagens (Pellegrino *et al.* 2005; Gehara *et al.* 2013; Amaral *et al.* 2013) já reportadas para esta região, sugere que esta área possa ter funcionado como um refúgio Pleistocénico.

Fig. 6.2 Mapa parcial do Brasil onde é indicada a região do vale do rio Paraíba do Sul e a serra do Mar coincidente com a distribuição geográfica dos haplótipos mitocondriais do clado BUR-RJ (traços amarelos). As localidades amostradas para a realização da Secção 3.3 encontram-se assinaladas com marcadores vermelhos. Escala: 1:60.000.000.

Este resultado vem assim corroborar hipóteses avançadas por outros autores, que sugerem que a atual biodiversidade da MA não pode ser explicada pelo um só modelo simplista (Carnaval & Moritz 2009), enfatizando a necessidade de estudos em uma escala mais fina (Martins 2011; Silva *et al.* 2012). Dentro desse contexto, destacam-se os organismos endémicos das regiões sudeste (como foi discutido acima) e, ainda, sul da MA. Estudos filogeográficos recentes tentaram cobrir esta lacuna ao analisar os padrões e os tempos de diversificação de organismos que só ocorrem na região sul da MA, tendo sugerido a presença de um refúgio putativo no Rio Grande do Sul (Valdez & D'Elía 2013) e até mesmo uma expansão demográfica anterior ao UMG (Batalha-filho *et al.* 2012). Estes resultados reforçam as evidências de persistência de habitats florestados na região sul da MA encontradas através de modelos de distribuição histórica e divergências profundas em anuros que ocorrem exclusivamente nesta região (Thomé *et al.* 2010).

Entre as espécies de *Phyllomedusa* do grupo *burmeisteri*, *P. tetraploidea*, *P. distincta* e *P. iheringii* são endémicas da região sul-sudeste da MA. Nesta tese, verificou-se que estas três espécies, filogeneticamente mais próximas entre si (grupo do sul) do que em relação às espécies distribuídas mais ao norte (grupo do norte) da MA (ver Secção 6.1), terão iniciado o processo de diversificação há aproximadamente ~2.5 milhões de anos (ver Secção 2.1). Estas três espécies apresentaram níveis elevados de subestruturação genética, que é particularmente evidente em *P. distincta* com a deteção de duas linhagens divergentes com uma distribuição geográfica coerente (*p*-não corrigida=1.5%). Interessante, foi a observação de níveis contrastantes de variabilidade genética entre estas duas linhagens. Sob o ponto de vista geográfico, a quebra entre a distribuição destas duas linhagens é coincidente com a posição do cisalhamento de Cubatão, como foi referido anteriormente (Fig. 6.1). Deste modo, com a finalidade de esclarecer os processos evolutivos responsáveis por esta subestruturação e diferentes níveis de variabilidade genética ao nível do mtDNA, (ver Secção 4.1), a amostragem desta espécie foi aumentada significativamente em relação à análise preliminar, tendo-se complementado a análise filogeográfica com base em métodos mais convencionais com o teste de hipóteses (teste de hipótese multilocus ABC) de diferentes cenários explicativos (filogeografia estatística), usando como marcadores genéticos um gene mitocondrial e três genes nucleares. Adicionalmente, os resultados genéticos foram contrastados com modelos de distribuição histórica projetados tanto para o Último

Interglacial (~120 mil anos) como para o Último Máximo Glacial (UMG; ~21 mil anos). No seu conjunto, o resultado destas análises suportam um cenário evolutivo de divergência antiga (~600 mil anos), seguida de uma recente expansão populacional (~6 mil anos) destas duas linhagens.

No seu conjunto, estes resultados reforçam a ideia de que o estudo dos processos e dos padrões de diversificação biológica na região Neotropical tem sido dificultado, principalmente, por três fatores. Primeiro, pela mudança constante de paradigmas, segundo pelo uso e abuso de generalizações a partir de poucos estudos usando metodologias similares e, finalmente, pela ausência de informação filogenética consistente para estimar tempos de diversificação (ver revisão em Rull 2013). É interessante aqui referir que muitas das generalizações evidenciam em certa medida uma tendência para estabelecer paralelos com os modelos elaborados em outras regiões do globo (Europa, América do Norte e a Austrália). No entanto, o atual nível de conhecimento dos padrões e hipóteses de diversificação biológica destas regiões é resultante de um lento e longo processo de investigação (ver referências em Weiss & Ferrand 2007; Shafer *et al.* 2010; Bell *et al.* 2011). Com efeito, desde os trabalhos pioneiros de Hewitt (1996, 2000) um conjunto cada vez mais relevante de trabalhos filogeográficos em organismos endémicos de regiões tradicionalmente encaradas como refúgio (e.g. Península Ibérica, Norte de África, Balcãs) sugere que estes cenários poderão ser demasiado simplistas para explicar os padrões atuais de variação genética. Por um lado, os mesmos três padrões descritos (1, isolamento em diferentes refúgios durante períodos glaciais e consequente diferenciação genética; 2, expansão pós-glacial recente; 3 miscigenação secundária após linhagens diferenciadas readquirirem contacto após a expansão) foram largamente descritos dentro dos Refúgios (o paradigma dos "refúgios dentro de refúgios"). Por outro lado, os organismos habitantes destas regiões apresentam também padrões de variação muito mais complexos e que não se enquadram totalmente com o modelo de resposta em 3 fases às oscilações climáticas, sugerindo em alternativa múltiplos pulsos de contração, expansão e miscigenação (Hewitt 1996, 2001; Gómez & Lunt 2007; Bell *et al*., 2011; Bryson *et al.* 2014).

6.3 Delimitação de espécies e implicações taxonómicas

Depois de muitos séculos de investigação é importante ressaltar que atualmente o campo da taxonomia, assim como o da biologia evolutiva em geral, tem passado por um importante processo integrativo de reavaliação conceptual e metodológica, principalmente influenciado pelos avanços da genética e da biologia molecular e técnicas computacionais de análise. Particularmente em regiões de elevada biodiversidade como as Tropicais e as Neotropicais, a utilização de abordagens integrativas tem-se revelado de extrema importância para a descoberta e descrição de novos taxa, assim como a reorganização da sistemática e taxonomia.

Nestas regiões biodiversas, a taxonomia de muitas espécies ainda é baseada unicamente em informações fenotípicas, que muitas vezes podem ser uma fonte de erro para a compreensão dos processos evolutivos e padrões biogeográficos históricos, especialmente entre espécies filogeneticamente relacionadas, devido ao conservadorismo ou à plasticidade fenotípica. Por exemplo, duas das cinco espécies de *Phyllomedusa* do grupo *burmeisteri* (*P. bahiana* e *P. burmeisteri*) apresentam um histórico taxonómico relativamente instável devido à existência de uma grande variação do fenótipo (ver detalhes na Secção 1.3.2). Deste modo, no Capítulo 3 procurou-se esclarecer a taxonomia destas espécies, aprofundando a investigação sobre o padrão de variação fenotípico e da variabilidade genética ao longo da área de distribuição das espécies. Com esta abordagem pretendeu-se averiguar se as formas que apresentam o padrão fenotípico intermediário são produto de hibridação extensa como foi anteriormente sugerido (Secção 3.1). Tanto os dados mitocondriais como os nucleares apoiaram o reconhecimento destas duas espécies como unidades taxonómicas com uma trajetória evolutiva independente. Os resultados mostraram ainda que a variação genética não foi congruente com a variação espacial dos padrões de coloração das superfícies ocultas da coxa (caracter fenotípico “diagnóstico”), variando consideravelmente dentro e entre populações de ambas as espécies. Este tipo de discordância já foi verificada em outras espécies da subfamília Phyllomedusinae (Ohmer *et al.* 2009; Bruschi *et al.* 2013; Brusa *et al.* 2013). Embora, existam sinais de mistura genética entre estas duas espécies, estes resultados não corroboraram a extensa área de variação clinal nordeste-sudeste anteriormente sugerida através da observação de indivíduos com padrão fenotípico intermediário (ver Secção 1.3.2).

De uma forma geral, os nossos resultados sugerem que o padrão espacial e a frequência da variação fenotípica da forma intermediária podem resultar de um processo de seleção natural agindo sobre as estratégias defensivas. Como foi referido na Secção 1.3.2, as espécies do género *Phyllomedusa*, apresentam padrões de coloração aposemáticos que são exibidos naturalmente quando estas se movem nos ramos das árvores estimuladas pela presença de potenciais predadores (ver Toledo *et al.* 2011 e Rudh & Qvarnström 2013). Com efeito, o ponto mais importante da teoria do aposematismo (Müller 1879) sugere que o fenótipo de populações de espécies distintas podem convergir em um padrão semelhante, pois desta forma o comportamento do predador será reforçado para evitar este padrão de coloração específico (por exemplo, ver Endler 1882; Harmon *et al.* 2005). Deste modo, é possível que a frequência mais elevada e a distribuição mais ampla da forma intermediária possa refletir o padrão de coloração com mais sucesso adaptativo. No entanto, atendendo à metodologia usada esta interpretação deverá ser encarada com precaução. A variabilidade intra-populacional dos padrões de coloração presentes em ambas as espécies merece um estudo mais aprofundado, com destaque particular para o papel da seleção sexual, características ecológicas e estratégias de comportamento destas espécies com características fenotípicas complexas.

Ainda neste capítulo foram utilizados métodos multilocus de agrupamento com base em frequências alélicas que não exigem informações *a priori*, redes de haplótipos multilocus e, ainda, abordagens com base no coalescente, como árvores de espécies e métodos recentes de delimitação de espécies (BPP), para testar estatisticamente a identificação de *P. burmeisteri, P. bahiana* e o clado BUR-RJ (linhagem altamente divergente revelada pelo mtDNA em *P. burmeisteri* localizada no estado do Rio de Janeiro, Secção 6.2) como unidades taxonómicas independentes. Em geral, as análises multilocus suportaram uma trajetória evolutiva independente tanto para as duas espécies previamente identificadas (*P. bahiana* e *P. burmeisteri*) como para a linhagem BUR-RJ. Adicionalmente, a árvore de espécie recuperou o relacionamento mais próximo entre *P. bahiana* e *P. burmeisteri*, e dois clados irmãos dentro de *P. burmeisteri* (BUR e BUR-RJ) independentemente do uso da informação dos dados de nuDNA ou quando mtDNA e nuDNA foram combinados.

diversidade, particularmente em casos de espécies com taxonomia complexa (Leaché & Fujita 2010; Fusinatto *et al.* 2013; Sousa-Neves *et al.* 2013; Satler *et al.* 2013), muito embora ainda existam desafios a serem ultrapassados, como por exemplo, a necessidade de definir a unidades *a priori*. Em particular, a separação de *P. burmeisteri* em duas unidades evolutivas (BUR e BUR-RJ) ainda carece de estudos adicionais, uma vez que esta separação não é totalmente congruente entre os diferentes tipos de marcadores e métodos (ver detalhes na Secção 3.1) ao contrário dos resultados para *P. bahiana* e *P. burmeisteri*. A este propósito, os resultados da análise da variabilidade genética de *P. burmeisteri* através de 11 microssatélites, especificamente desenvolvidos para esta espécie (ver detalhes na Secção 3.2), mostraram a existência de um grupo geneticamente diferenciado restrito ao estado de Rio de Janeiro (ver detalhes na Secção 3.3), parcialmente coincidente com a distribuição do clado BUR-RJ. No entanto, enquanto a divergência entre BUR-RJ é aproximadamente seis vezes maior do que as divergências encontradas entre os demais clados mitocondriais, para os microssatélites as divergências (FST e Da) foram semelhantes entre os quatro grupos genéticos (clusters). É possível que as rápidas taxas de mutação, tipicamente observados em microssatélites, aliadas à homoplasia e à ocorrência de fluxo gênico contemporâneo possam explicar esta discordância.

Por fim, é importante ressaltar que a investigação do *status* taxonómico desta nova unidade evolutiva (BUR-RJ) pode ainda beneficiar de outras abordagens integrativas, incluindo a análise de aspetos reprodutivos, como por exemplo dos cantos de acasalamento e, ainda, de estudos de morfometria geométrica para a comparação de marcos anatómicos. Curiosamente, a localidade tipo de *P. burmeisteri* é a cidade do Rio de Janeiro, o que em caso de reconhecimento taxonómico desta nova unidade evolutiva como espécie, implicaria a atribuição de um novo epíteto específico a *P. burmeisteri* distribuída fora do estado do Rio de Janeiro.

6.4 Modelos de nicho ecológico

As diversas aplicações dos modelos de nicho ecológico (ENM) no campo da filogeografia têm sido amplamente comprovadas (ver revisão em Alvarado-Serrano & Knowles 2014). Nesta tese, esta ferramenta foi utilizada de duas formas: 1) para

prever a distribuição potencial de *P. distincta* no passado como fonte de validação externa para os resultados obtidos a partir do teste de hipóteses de cenários evolutivos (Secção 4.1) e; 2) para comparar o nicho e a influência das condições ambientais na distribuição de espécies diploides (*P. distincta* e *P. iheringii*) e poliploide (*P. tetraploidea*) (Secção 5.1).

No caso da espécie *P. distincta*, a utilização da modelação paleo-climática combinada com uma amostragem extensa ao longo de toda a sua área de distribuição e análises multilocus, incluindo o teste de hipóteses de cenários evolutivos usando uma análise ABC (Secção 4.1), foi essencial para a elaboração de hipóteses explicativas sobre a história evolutiva das duas linhagens altamente divergentes ao nível do mtDNA (Secção 2.2). Os resultados obtidos neste estudo mostraram, pela primeira vez, que o extremo sul da zona de cisalhamento de Cubatão, perto das transições entre os estados do Paraná e Santa Catarina, poderá estar associado a uma ruptura filogeográfica na MA (Fig. 6.1), evidência que, de uma maneira geral, parece ser apoiada pelo teste de hipóteses. Com efeito, a hipótese que melhor se adequa aos dados favoreceu um cenário de vicariância antiga (Pleistoceno Médio), com uma expansão populacional moderada durante o Holoceno. De fato, o modelo projetado para o UMG corroborou a hipótese de fragmentação da distribuição de *P. distincta* no passado revelando duas áreas de refúgio potencial, sendo uma área isolada no litoral do estado do PR e outra área isolada próxima ao estado de SC, mas que hoje em dia é coberta pelo Oceano Atlântico. É possível que, os resultados da modelação histórica da distribuição desta espécie possam refletir o impacto das oscilações climáticas do final do Pleistoceno na diversificação de *P. distincta*. É importante referir que, pelo facto destes resultados poderem ser influenciados pela baixa resolução dos modelos paleo-climáticos de circulação global da atmosfera-oceano referentes à América do Sul (ver Rojas *et al*. 2009; Collevatti *et al*. 2013), optou-se por não usar as previsões da modelação ao detalhe para corroborar os cenários demográficos hipotéticos, embora os mesmos se mostrem informativos considerando que a distribuição de *P. distincta* possa ter sofrido os efeitos das alterações climáticas no passado.

A combinação de ferramentas moleculares e ENM já vem sendo utilizada para descrever as hipóteses de diversificação de anfíbios na MA e os resultados são variados (ver Carnaval *et al*. 2009; Thomé *et al*. 2010). No entanto, este estudo foi o primeiro a utilizar esse tipo de combinação com uma espécie endémica do sudeste

da MA, região considerada como instável durante períodos glaciais e, por conseguinte, uma área de colonização recente. Deste modo, este estudo fornece uma nova perspetiva sobre a região sudeste da MA que requer mais estudos detalhados num futuro próximo.

Por outro lado, o primeiro grande objetivo da análise dos ENM's, das duas espécies diploides e da poliploide, do grupo *P. burmeisteri* foi identificar as principais variáveis ecogeográficas que explicam a distribuição potencial destas espécies. Este estudo destaca-se por representar o primeiro trabalho que combina um conjunto abrangente de variáveis climáticas, topográficas e de habitat para explorar tanto o espaço geográfico como o ecológico de grupo de anfíbios diploide-polipoide. Em particular, as análises baseadas em uma escala fina do GIS foram úteis para fornecer novas evidências sobre a origem da poliploidia e na identificação de fatores que afetam tanto localização e manutenção de zonas híbridas naturais neste grupo de sapos folha. Os modelos de máxima entropia sugeriram que a distribuição destas três espécies dependem mais de fatores bioclimáticos do que do tipo de habitat. Embora as espécies do gênero *Phyllomedusa* ocorram predominantemente em áreas florestadas próximas a massas de água e apresentem um modo de reprodução com base em ninhos feitos em folhas pendentes sobre a água (Modo de 24 em Haddad & Prado 2005), observações feitas em campo sugerem que estas espécies podem se adaptar a perturbações antrópicas moderadas. Por exemplo, *P. distincta* já foi encontrado se reproduzindo em charcos localizados em eucaliptais e *P. iheringii* em culturas de soja (TOB e JA observações pessoais, respetivamente).

Ambos os tipos de monoculturas, entre outras, estão em rápida expansão na Mata Atlântica e veem sendo considerados como uma das principais ameaças para a conservação da biodiversidade deste bioma (Ribeiro *et al.* 2011b). No entanto, embora estes sapos folha possam se reproduzir utilizando as folhas das plantas acima referidas, a diminuição da conectividade entre os remanescentes florestais e os efeitos de bordadura adicionais, em particular no caso de zonas agrícolas, podem atuar negativamente sobre a variabilidade genética dessas espécies, reduzindo o fluxo gênico entre as populações.

Em segundo lugar, procurou-se investigar se existem diferenças entre o nicho das espécies diploides e da poliploide, uma vez que a distribuição das espécies poliploides tem sido associada a habitats mais severos (altitudes elevadas e clima árido) quando comparados com o nicho ocupado pelas espécies progenitoras. De

fato, neste estudo verificou-se que existe diferenciação entre os nichos das três espécies analisadas. No entanto, em todas as comparações utilizadas, os resultados foram congruentes em mostrar que o nicho mais divergente é entre a diploide *P. iheringii* e a poliploide *P. tetraploidea*. Além disso, verificou-se, que a espécie poliploide tende a ocorrer em áreas com elevada amplitude térmica e evapotranspiração potencial quando comparado com as duas espécies diploides. Embora as espécies do gênero *Phyllomedusa* sejam considerados "sapo à prova de água", por apresentarem adaptações eco-fisiológicas ligadas a resistência à dessecação (ver Shoemaker *et al*, 1972; Faivovich *et al*, 2010), *P. tetraploidea* parece beneficiar de seu genoma duplicado para adaptar-se a habitats mais severos. Esta hipótese não corrobora as observações de Mable *et al*. (2011) feita com base em vários anfíbios e peixes. Estes autores, utilizando a classificação das zonas climáticas de Köppen, não encontraram diferenças entre as condições de habitat entre espécies diploides e poliploides. É possível, no entanto, que este resultado se deva à falta de resolução da classificação de Köppen em escala fina.

Com efeito, Otto *et al*. (2007), utilizando uma abordagem semelhante a utilizada neste trabalho, encontraram os mesmos indícios gerais relatados para *P. tetraploidea*. Não obstante, também é possível que esta ocorrência em habitats mais severos por parte da espécie tetraploide possa refletir a sua filogenia, em vez de adaptação devido à poliploidia (e.g. Martin & Husband, 2009).

Por último, procurou-se identificar áreas de potencial simpatria entre pares de espécies através da sobreposição dos modelos de distribuição potencial e, ainda, verificar se estas áreas correspondem a áreas de transição ambiental suaves ou abruptas. Deste modo, foram previstas duas áreas de potencial simpatria, uma entre as duas espécies diploides (*P. distincta* e *P. iheringii*) (SSA, Sympatric South area) e outra entre a espécie diploide, *P. distincta*, e a espécie poliploide (SSN, Sympatric North area). A análise a uma escala fina de ambas as áreas sugeriu que apenas a zona híbrida natural, descrita anteriormente entre *P. tetraploidea* e *P. distincta* (Haddad *et al*. 1994; Gruber *et al*. 2013), está localizada em uma área de transição suave, enquanto a zona de potencial simpatria prevista entre as duas espécies diploides exibe variações abruptas. Este último resultado sugere que existe uma possibilidade de hibridação extremamente reduzida entre as espécies diploides. No entanto, trabalhos de campo futuros na região da SSA poderão esclarecer esta hipótese.

Muito embora ainda existam desafios consideráveis referente a qualidade dos dados dos modelos paleoclimáticos de circulação global da atmosfera-oceano disponíveis atualmente para a região da América do Sul, em conjunto, ambas as utilizações dos ENM no contexto geral desta tese foram essenciais para esclarecer diversas questões relacionados com a história evolutiva das espécies de *Phyllomedusa* do grupo *burmeisteri* endêmicas do sudeste da América do Sul. Neste contexto, interessa realçar a sua importância para o estudo de vários aspetos associados com a poliploidização, considerando que cerca de dez casos de poliploidia (ver referências em Bogart 1980; Mable *et al*. 2011) já foram descritos em várias espécies de anuros endêmicos do América do Sul, esta abordagem pode especialmente ser vista como um ponto de partida para futuros estudos comparativos sobre o papel da biogeografia na origem, evolução e distribuição de espécies poliploide.

6.5 Considerações finais e perspetivas futuras

O conjunto de artigos apresentados nesta tese permitiu aprofundar de um modo muito significativo o conhecimento sobre a taxonomia e as relações evolutivas e os processos de diversificação das espécies de *Phyllomedusa* do grupo *burmeisteri*. Isto só foi possível devido à utilização de uma densa amostragem para todas as espécies do grupo, o uso de vários tipos de marcadores moleculares (mtDNA, nuDNA e microssatélites) e fenotípicos e, ainda, a utilização de vários métodos e ferramentas de análise. Entre estes destacam-se o uso de métodos recentemente desenvolvidos para a testar a delimitação de espécies, métodos de teste de hipóteses sobre cenários filogeográficos através da Aproximação Bayesiana e ainda ferramentas como, por exemplo, os modelos de nicho ecológico.

Apesar da relevância do conjunto de dados obtidos, muitos aspetos sobre a história evolutiva deste grupo necessitam de uma investigação adicional mais aprofundada, quer aumentando a amostragem, quer utilizando um número mais elevado de marcadores moleculares. A este respeito importa salientar a zona hibrida entre *P. burmeisteri* e *P. bahiana*, em que a reduzida amostragem e a ausência de conhecimentos sobre parâmetros-chave, como os padrões de introgressão para diferentes loci, a força e o modo de atuação da seleção natural, a influência de barreiras pré- e pós-zigóticas *versus* parâmetros demográficos relacionados com

fatores recentes e históricos (p. ex. oscilações climáticas do Pleistoceno) nos padrões de introgressão, constituem lacunas que deverão ser colmatas em futuros estudos. Neste contexto, refira-se o desenvolvimento de um número muito significativo de SNPs, atualmente possível através da utilização de técnicas recentes de sequenciação de nova geração (ver Ellegren 2014). Adicionalmente, o recurso a cruzamentos controlados em laboratório poderá responder a questões-chave, nomeadamente quanto à fertilidade e viabilidade dos híbridos e ainda aspetos relacionados com a preferência sexual, aspeto que se reveste da maior importância para entender possíveis assimetrias na direção da introgressão. Por fim, a elevada variação geográfica do padrão fenotípico (padrão de coloração das partes ocultas da coxa) observado em ambas as espécies, com a deteção de vários tipos intermédios entre os padrões típicos previamente atribuídos a cada uma das espécies, merece uma análise mais detalhada no futuro. Isto é particularmente importante atendendo ao papel evolutivo da coloração em vários grupos de anfíbios, quer em mecanismos de defesa, quer em aspetos reprodutivos.

Tal como foi já acima referido, o uso de modelos de nicho ecológico foi crucial para a formulação de algumas hipóteses explicativas de processos evolutivos, nomeadamente para a interpretação da história evolutiva de *P. distincta*. Assim, no futuro será útil estender a utilização desta ferramenta para o estudo da história evolutiva de *P. burmeisteri* e *P. bahiana*. No que diz respeito a esta ferramenta, é ainda importante salientar a sua importância para a comparação de nicho entre as espécies diploides (*P. distincta* e *P. iheringii*) e entre estas e a forma tetraploide (*P. tetraploidea*). Embora os modelos de nicho ecológico tenham sido já explorados em alguns trabalhos recentes, o tipo de abordagem efetuado neste estudo, combinando análises multivariadas com a aplicação deste tipo de modelos a uma escala fina para a análise de diversas variáveis climáticas, tipo de habitat e declive, pode ser considerada pioneira. Os resultados obtidos não só forneceram dados importantes sobre as diferenças entre os nichos destas espécies com diferentes níveis de ploidia, como forneceram evidências sobre a origem da espécie tetraploide.

No entanto, trabalhos de investigação futuros que incluam a análise de nicho das demais espécies do género *Phyllomedusa* num contexto filogenético poderão reforçar e fornecer evidências adicionais às hipóteses já propostas neste estudo. Além disso, a projeção da distribuição das espécies diploides e tetraploide para o passado poderá fornecer informações adicionais sobre a origem da espécie

tetraploide. A este propósito, é importante referir que atualmente já existem enquadramentos analíticos que ultrapassam um dos principais problemas da modelação paleoclimática, representado por um constrangimento relacionado com a estabilidade do nicho ao longo do tempo (e.g. Takahashi *et al.* 2014). Além disso, a modelação destas espécies também para o futuro, poderia ajudar a responder questões sobre os potenciais benefícios da poliploidia frente às previsões de aquecimento global.

Em conjunto, as investigações futuras sobre as diversas questões levantadas por esta tese poderão vir a contribuir significativamente para o aprofundamento de temas centrais da biologia evolutiva, nomeadamente para a compreensão dos processos de especiação gradual e instantâneo, assim como para o estabelecimento de medidas de conservação dos mesmos.

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Anexos

Anexo A

Material suplementar da Secção 2.1

Table A.1 Population number (code), taxon, voucher (see text for institution information) and mitochondrial haplotypes of *Phyllomedusa burmeisteri* species group."*", tissue samples without vouchers.

Code	Taxon	Voucher	*h*
1	*P. bahiana*	CFBH 2596	1
2	*P. bahiana*	CFBH 19514, 19524	2
2	*P. bahiana*	CFBH 19525, 19526	3
3,4	*P. bahiana*	CFBH 18741, 18773, 13355,13357, 13359, 13360	4
3	*P. bahiana*	CFBH 18747	5
3,4	*P. bahiana*	CFBH 18772, 13358	6
5	*P. bahiana*	CFBH *	7
6	*P. bahiana*	CFBH *	8
7	*P. bahiana*	CFBH 10207, 10209, 10210	9
8	*P. burmeisteri*	CFBH *	10
9	*P. burmeisteri*	CFBH 10207, 10209, 10210	11
10	*P. burmeisteri*	CFBH 14428	12
11	*P. burmeisteri*	CFBH *	13
12,15, 18,21	*P. distincta*	CFBH 8334, 8246, 2657, 2659, MTR *	14
13	*P. tetraploidea*	MZUSP A 134841, 134839	15
13	*P. tetraploidea*	MZUSP A 134838	16
13,15	*P. tetraploidea, distincta*	MZUSP A 134840, CFBH 2125, 8249	17
14	*P. tetraploidea*	CFBH 18829	18
15,19	*P. distincta, tetraploidea*	CFBH 8245, * , MZUSP A 129325, CFBH 2096	19
15	*P. distincta*	CFBH 8250, 8251, 8252, 2047	20
15	*P. tetraploidea*	CFBH *	21
15	*P. tetraploidea*	CFBH 2126	22
15,17	*P. distincta*	CFBH 2114, *	23
16	*P. distincta*	CFBH 13868	24
20	*P. distincta*	CFBH 11067	25
21	*P. distincta*	CFBH 2658	26
22,24, 27	*P. distincta*	CFBH 10998, 10999, 10990, 11007, 10321, 12408, 12409, 12410, 12412	27
23	*P. tetraploidea*	CFBH 18251, 18253, 18254	28
23	*P. tetraploidea*	CFBH 18258	29
25	*P. tetraploidea*	CFBH *	30
26	*P. tetraploidea*	CFBH 6949, 6950	31
28,29	*P. iheringii*	CFBH *	32
28	*P. iheringii*	CFBH 3894	33
29	*P. iheringii*	CFBH *	34
30	*P. iheringii*	MCT/PUCRS 4238	35
-	*P. boliviana*	CFBH 2570	-

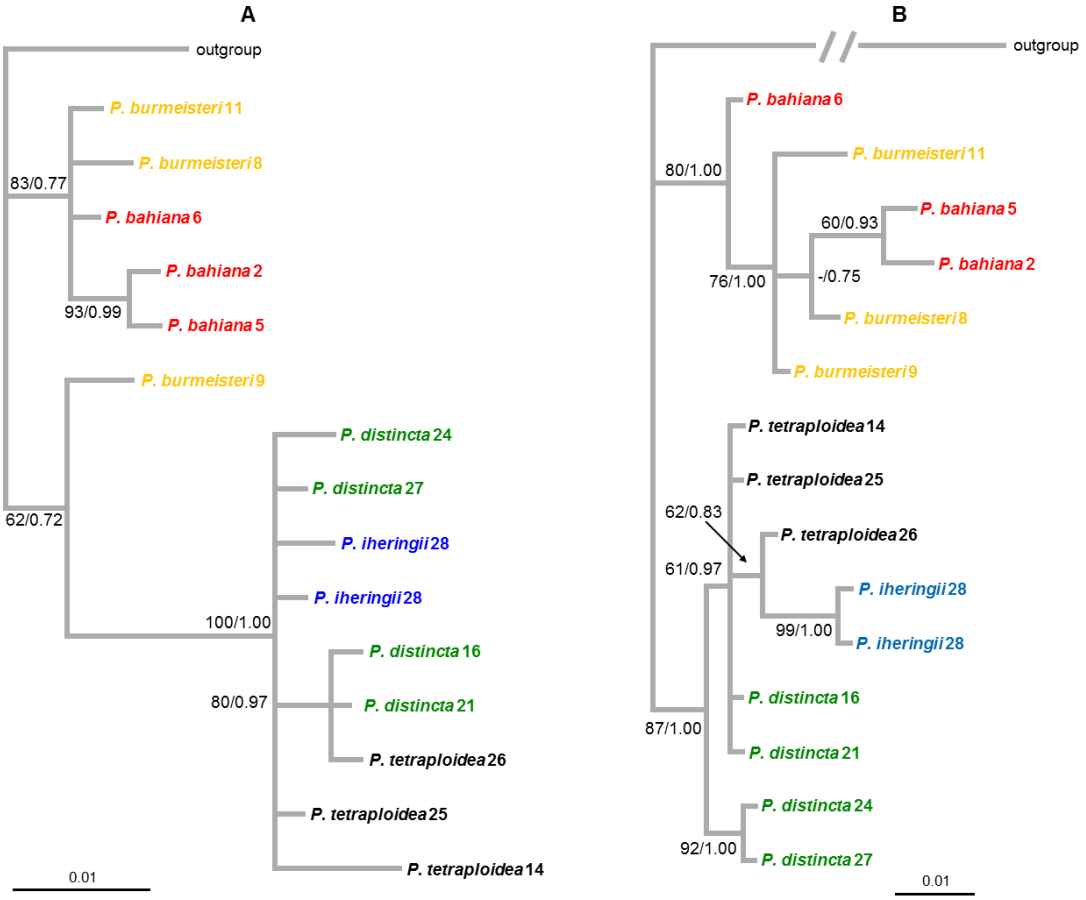

Fig. A.1 Tree derived from Bayesian analysis of (data set 1) 454 of the C-myc2 (A) and 604bp b-fibint7 (B) nuclear sequences in the *Phyllomedusa burmeisteri* species group. Bootstrap values ML and Bayesian posterior probabilities are given near the branches, respectively. Values under 50% are represented by "–".

Anexo B

Material suplementar para a Secção 3.1

Table B.1 Individual geographic, phenotypic, and genetic sample information of *Phyllomedusa bahiana and Phyllomedusa burmeisteri*. Morphotype classification: S = *Phyllomedusa burmeisteri*, I = "intermediate", and U = *Phyllomedusa bahiana*.

N	LC	Lab nº	Locality	ES	Voucher nº	Tissue nº	**Morph.**	ND2	STRUC.	C-myc2	β-fibin7	CXCR4
1	1	**1***	Areia Branca	SE	CFBH 2596	CFBH-T 162	**U**	BAH ([b]HQ262457.1)	BAH	KM365615	KM365560	KM365681
2	2	**292***	Indiaroba	SE	MNRJ 49741		**I**	BAH (KM365811)	BAH	KM365616	KM365561	KM365682
3	2	**293**	Indiaroba	SE	MNRJ 46750		**U**	BAH (KM365812)	-	-	-	-
4	3	**380**	Acajutiba	BA	CFBH 27949	CFBH-T 13463	**U**	BAH (KM365813)	-	-	-	-
5	3	**381***	Acajutiba	BA	CFBH 27952	CFBH-T 13466	**U**	BAH (KM365814)	BAH	KM365617	KM365562	KM365683
6	3	**382**	Acajutiba	BA	CFBH 27953	CFBH-T 13467	**U**	BAH (KM365815)	-	-	-	-
7	3	**383**	Acajutiba	BA	CFBH 27954	CFBH-T 13468	**U**	BAH (KM365816)	-	-	-	-
8	4	**606**	Itaitú	BA	CFBH 34588	CFBH-T 17839	**U**	BAH (KM365817)	-	-	-	-
9	4	**607**	Itaitú	BA	CFBH 34589	CFBH-T 17840	**U**	BAH (KM365818)	-	-	-	-
10	4	**608**	Itaitú	BA	CFBH 34590	CFBH-T 17841	**U**	BAH (KM365819)	-	-	-	-
11	4	**609**	Itaitú	BA	CFBH 34591	CFBH-T 17842	**U**	BAH (KM365820)	-	-	-	-
12	4	**610**	Itaitú	BA	CFBH 34592	CFBH-T 17843	**U**	BAH (KM365821)	-	-	-	-
13	4	**611**	Itaitú	BA	CFBH 34593	CFBH-T 17844	**U**	BAH (KM365822)	-	-	-	-
14	4	**612**	Itaitú	BA	CFBH 34594	CFBH-T 17845	**U**	BAH (KM365823)	-	-	-	-
15	4	**613**	Itaitú	BA	CFBH 34595	CFBH-T 17846	**U**	BAH (KM365824)	-	-	-	-
16	4	**614**	Itaitú	BA	CFBH 34615	CFBH-T 17847	**U**	BAH (KM365825)	-	-	-	-
17	5	**447**	Castro Alves	BA	MHNJCH 141		**U**	BAH (KM3658269)	BAH	KM365618	KM365563	-
18	5	**449***	Castro Alves	BA	MHNJCH 144		**I**	BAH (KM365827)	BAH	KM365619	KM365564	KM365684
19	6	**450**	Cachoeira	BA	MHNJCH 156		**U**	BAH (KM365828)	BAH	-	-	KM365685
20	6	**451***	Cachoeira	BA	MHNJCH 157		**U**	BAH (KM365829)	BAH	-	KM365565	KM365686
21	6	**452***	Cachoeira	BA	MHNJCH 158		**U**	BAH (KM365830)	BAH	-	KM365566	KM365687
22	6	**453**	Cachoeira	BA	MHNJCH 159		**I**	BAH (KM365831)	-	-	-	-
23	6	**454**	Cachoeira	BA	MHNJCH 160		**U**	BAH (KM365832)	-	-	-	-
24	6	**455**	Cachoeira	BA	MHNJCH 161		**U**	BAH (KM365833)	-	-	-	-
25	6	**456**	Cachoeira	BA	MHNJCH 162		**U**	BAH (KM365834)	-	-	-	-
26	6	**457**	Cachoeira	BA	MHNJCH 163		**U**	BAH (KM365835)	-	-	-	-
27	7	**389**	Dias D'Ávila	BA	CFBH 27992	CFBH-T 13506	**I**	BAH (KM365836)	-	-	-	-
28	7	**390***	Dias D'Ávila	BA	CFBH 27993	CFBH-T 13507	**U**	BAH (KM365837)	BAH	KM365620	KM365567	KM365688
29	8	**436**	Amargosa	BA	UFBA 8333	UFBA-T 391	**U**	BAH (KM365838)	-	-	-	-
30	8	**438**	Amargosa	BA	UFBA 7303	UFBA-T 263	**I**	BAH (KM365839)	-	-	-	-
31	8	**439**	Amargosa	BA	UFBA 7304	UFBA-T 262	**I**	BAH (KM365840)	-	-	-	-
32	9	**458**	Santo Antônio de Jesus	BA	MHNJCH 217		**I**	BAH (KM365841)	-	-	-	-
33	9	**459**	Santo Antônio de Jesus	BA	MHNJCH 218		**U**	BAH (KM365842)	-	-	-	-
34	9	**460**	Santo Antônio de Jesus	BA	MHNJCH 219		**U**	BAH (KM365843)	BAH	-	-	KM365689
35	9	**461**	Santo Antônio de Jesus	BA	MHNJCH 220		**U**	BAH (KM365844)	BAH	-	-	KM365690
36	10	**446**	Contendas do Sincorá	BA	MHNJCH 222		**U**	BAH (KM365845)	-	-	-	-
37	11	**569**	Mucugê	BA	CFBH 30096	CFBH-T 15760	**U**	BAH (KM365846)	-	-	-	-
38	11	**570**	Mucugê	BA	CFBH 30097	CFBH-T 15761	**U**	BAH (KM365847)	-	-	-	-
39	11	**571**	Mucugê	BA	CFBH 30098	CFBH-T 15762	**U**	BAH (KM365848)	-	-	-	-
40	11	**572**	Mucugê	BA	CFBH 30099	CFBH-T 15763	**I**	BAH (KM365849)	-	-	-	-
41	11	**573**	Mucugê	BA	CFBH 30100	CFBH-T 15764	**I**	BAH (KM365850)	-	-	-	-
42	11	**574**	Mucugê	BA	CFBH 30101	CFBH-T 15765	**U**	BAH (KM365851)	-	-	-	-
43	11	**575**	Mucugê	BA	CFBH 30102	CFBH-T 15766	**U**	BAH (KM365852)	-	-	-	-
44	11	**576**	Mucugê	BA	CFBH 30103	CFBH-T 15767	**U**	BAH (KM365853)	-	-	-	-
45	11	**577**	Mucugê	BA	CFBH 30104	CFBH-T 15768	**U**	BAH (KM365854)	-	-	-	-
46	11	**578**	Mucugê	BA	CFBH 30105	CFBH-T 15769	**U**	BAH (KM365855)	-	-	-	-
47	12	**513**	Valença	BA	MHNJCH 251		**U**	BAH (KM365856)	-	-	-	-
48	12	**514**	Valença	BA	MHNJCH 252		**I**	BAH (KM365857)	BAH	KM365621	-	-
49	13	**567**	Riacho Santana	BA	UFMG-A 4833	UFMG-T 1470b	**I**	BAH (KM365858)	-	-	-	-
50	14	**240**	Caetité	BA	CFBH 22159	CFBH-T 11034	**U**	BAH (KM365859)	-	-	-	-

N	LC	Lab nº	Locality	ES	Voucher nº	Tissue nº	Morph.	ND2	STRUC.	C-myc2	β-fibin7	CXCR4
51	14	**241**	Caetité	BA	CFBH 22160	CFBH-T 11036	-	BAH (KM365860)	-	-	-	-
52	14	**242**	Caetité	BA	CFBH 22158	CFBH-T 11091	U	BAH (KM365861)	-	-	-	-
53	15	**243**	Livramento de Nª Senhora	BA	CFBH 23362	CFBH-T 11064	U	BAH (KM365862)	BAH	KM365622	KM365568	-
54	16	**566**	Jacaraci	BA	UFMG-A 4774	UFMG-T 1449b	U	BAH (KM365863)	-	-	-	-
55	17	**20**	Grão Mogol	MG	CFBH 10207	CFBH-T 2372	I	BAH ([b]HQ262465.1)	BAH	-	-	KM365691
56	17	**21**	Grão Mogol	MG	CFBH 10210	CFBH-T 2387	I	BAH (KM365864)	BAH	KM365623	KM365569	KM365692
57	17	**27**	Grão Mogol	MG	CFBH 10208	CFBH-T 2382	I	BAH (KM365865)	BAH	-	-	KM365693
58	17	**29**	Grão Mogol	MG	CFBH 10209	CFBH-T 2393	I	BAH (KM365866)	-	-	-	-
59	18	**108**	Maracás	BA	UFBA 2295	UFBA-T 44	U	-	BAH	KM365624	-	KM365694
60	18	**110**	Maracás	BA	UFBA 2297	UFBA-T 46	U	BAH (KM365867)	-	-	-	-
61	18	**111**	Maracás	BA	UFBA 2298	UFBA-T 47	U	BAH (KM365868)	-	-	-	-
62	18	**112**	Maracás	BA	UFBA 2299	UFBA-T 48	U	BAH (KM365869)	-	-	-	-
63	18	**113**	Maracás	BA	UFBA 2300	UFBA-T 49	U	BAH (KM365870)	BAH	-	-	KM365695
64	18	**114**	Maracás	BA	UFBA 2301	UFBA-T 50	U	-	BAH	KM365625	-	KM365696
65	18	**115**	Maracás	BA	UFBA 2302	UFBA-T 51	U	BAH (KM365871)	BAH	KM365626	-	KM365697
66	18	**183**	Maracás	BA	CFBH 19514	CFBH-T 9371	U	BAH ([b]HQ262458.1)	BAH	-	-	KM365698
67	18	**184**	Maracás	BA	CFBH 19521	CFBH-T 9378	U	BAH (KM365872)	-	-	-	-
68	18	**185**	Maracás	BA	CFBH 19522	CFBH-T 9379	U	BAH (KM365873)	-	-	-	-
69	18	**186**	Maracás	BA	CFBH 19524	CFBH-T 9381	U	BAH (KM365874)	-	-	-	-
70	18	**187**	Maracás	BA	CFBH 19525	CFBH-T 9382	-	BAH (KM365875)	-	-	-	-
71	18	**188**	Maracás	BA	CFBH 19526	CFBH-T 9383	U	BAH ([b]HQ262459.1)	BAH	[b]HQ262443.1	[b]HQ262427.1	-
72	18	**189**	Maracás	BA	CFBH 19539	CFBH-T 9396	U	BAH (KM365876)	-	-	-	-
73	19	**425**	Jequié	BA	MHNJCH 56		I	BAH (KM365877)	BAH	KM365627	-	-
74	19	**426**	Jequié	BA	MHNJCH 57		I	BAH (KM365878)	BAH	KM365628	KM365570	-
75	19	**427**	Jequié	BA	MHNJCH 65		I	BAH (KM365879)	-	-	-	-
76	19	**428**	Jequié	BA	MHNJCH 82		I	BAH (KM365880)	-	-	-	-
77	19	**429**	Jequié	BA	MHNJCH 83		U	BAH (KM365881)	BAH	KM365629	KM365571	-
78	19	**430***	Jequié	BA	MHNJCH 84		U	BAH (KM365882)	BAH	KM365630	KM365572	KM365699
79	19	**431**	Jequié	BA	MHNJCH 122		I	BAH (KM365883)	-	-	-	-
80	19	**432**	Jequié	BA	MHNJCH 134		I	BAH (KM365884)	BAH	-	-	KM365700
81	19	**433**	Jequié	BA	MHNJCH 135		I	BAH (KM365885)	-	-	-	-
82	19	**517**	Jequié	BA	MHNJCH 293		I	BAH (KM365886)	-	-	-	-
83	19	**522**	Jequié	BA	MHNJCH 302		U	BAH (KM365887)	-	-	-	-
84	19	**523**	Jequié	BA	MHNJCH 303		I	BAH (KM365888)	-	-	-	-
85	19	**524**	Jequié	BA	MHNJCH 305		U	BAH (KM365889)	-	-	-	-
86	19	**525**	Jequié	BA	MHNJCH 340		I	BAH (KM365890)	-	-	-	-
87	20	**384**	Gandú	BA	CFBH 27977	CFBH-T 13491	I	BAH (KM365891)	-	-	-	-
88	20	**385**	Gandú	BA	CFBH 27978	CFBH-T 13492	I	BAH (KM365892)	-	-	-	-
89	20	**386**	Gandú	BA	CFBH 27979	CFBH-T 13493	U	BAH (KM365893)	-	-	-	-
90	20	**387**	Gandú	BA	CFBH 27980	CFBH-T 13494	I	BAH (KM365894)	-	-	-	-
91	20	**388**	Gandú	BA	CFBH 27981	CFBH-T 13495	I	BAH (KM365895)	-	-	-	-
92	20	**512**	Gandú	BA	MHNJCH 250		I	BAH (KM365896)	-	-	-	-
93	20	**515**	Gandú	BA	MHNJCH 291		I	BAH (KM365897)	-	-	-	-
94	20	**516**	Gandú	BA	MHNJCH 292		I	BAH (KM365898)	-	-	-	-
95	20	**518**	Gandú	BA	MHNJCH 295		I	BAH (KM365899)	-	-	-	-
96	20	**519**	Gandú	BA	MHNJCH 296		I	BAH (KM365900)	-	-	-	-
97	20	**521**	Gandú	BA	MHNJCH 298		U	BAH (KM365901)	-	-	-	-
98	21	**437**	Ituberá	BA	UFBA 10459	UFBA-T 506	I	BAH (KM365902)	-	-	-	-
99	22	**406**	Igrapiúna	BA	-	CFBH-T 13534	-	BAH (KM365903)	-	-	-	-
100	22	**407**	Igrapiúna	BA	-	CFBH-T 13535	-	BAH (KM365904)	-	-	-	-
101	22	**408**	Igrapiúna	BA	-	CFBH-T 13536	-	BAH (KM365905)	-	-	-	-
102	22	**409**	Igrapiúna	BA	-	CFBH-T 13537	-	BAH (KM365906)	-	-	-	-
103	22	**410**	Igrapiúna	BA	-	CFBH-T 13538	-	BAH (KM365907)	-	-	-	-
104	22	**411**	Igrapiúna	BA	-	CFBH-T 13539	-	BAH (KM365908)	-	-	-	-
105	22	**412**	Igrapiúna	BA	-	CFBH-T 13540	-	BAH (KM365909)	BAH	KM365631	-	-
106	22	**413**	Igrapiúna	BA	-	CFBH-T 13541	-	BAH (KM365910)	BAH	KM365632	KM365573	-
107	23	**391**	Camamu	BA	CFBH 27997	CFBH-T 13511	I	BAH (KM365911)	BAH	KM365633	KM365574	-
108	23	**392**	Camamu	BA	CFBH 27998	CFBH-T 13512	U	BAH (KM365912)	BAH	KM365634	-	-
109	23	**393**	Camamu	BA	CFBH 27999	CFBH-T 13513	I	BAH (KM365913)	-	-	-	-
110	24	**394**	Itacaré	BA	CFBH 28000	CFBH-T 13514	I	BAH (KM365914)	-	-	-	-

N	LC	Lab nº	Locality	ES	Voucher nº	Tissue nº	Morph.	ND2	STRUC.	C-myc2	β-fibin7	CXCR4
111	24	**395**	Itacaré	BA	CFBH 28001	CFBH-T 13515	**I**	BAH (KM365915)	BAH	KM365635	-	KM365701
112	25	**171**	Aurelino Leal	BA	CFBH 18741	CFBH-T 9256	**U**	BAH ([b]HQ262460.1)	-	-	-	-
113	25	**172**	Aurelino Leal	BA	CFBH 18747	CFBH-T 9261	**I**	BAH ([b]HQ262461.1)	BAH	KM365636	-	KM365702
114	25	**173**	Aurelino Leal	BA	CFBH 18748	CFBH-T 9262	**I**	BAH (KM365916)	-	-	-	-
115	25	**174**	Aurelino Leal	BA	CFBH 18771	CFBH-T 9283	**I**	BAH (KM365917)	BAH	-	-	KM365703
116	25	**175**	Aurelino Leal	BA	CFBH 18772	CFBH-T 9282	**U**	BAH ([b]HQ262462.1)	BAH	-	-	-
117	25	**176**	Aurelino Leal	BA	CFBH 18773	CFBH-T 9284	**I**	BAH (KM365918)	-	-	-	-
118	26	**5**	Uruçuca	BA	CFBH 13355	CFBH-T 4127	**I**	BAH (KM365919)	BAH	-	-	KM365704
119	26	**6**	Uruçuca	BA	CFBH 13356	CFBH-T 4128	**S**	BAH (KM365920)	BAH	-	-	KM365705
120	26	**7**	Uruçuca	BA	CFBH 13357	CFBH-T 4134	**I**	BAH (KM365921)	-	-	-	-
121	26	**8**	Uruçuca	BA	CFBH 13358	CFBH-T 4135	**I**	BAH (KM365922)	BAH	KM365637	-	KM365706
122	26	**9**	Uruçuca	BA	CFBH 13359	CFBH-T 4136	**I**	BAH (KM365923)	BAH	-	-	KM365707
123	26	**10**	Uruçuca	BA	CFBH 13360	CFBH-T 4137	**I**	BAH (KM365924)	BAH	-	-	KM365708
124	26	**107**	Uruçuca	BA	CFBH 13354	CFBH-T 4147	**I**	BAH (KM365925)	BAH	KM365638	KM365575	KM365709
125	26	**396**	Uruçuca	BA	CFBH 28004	CFBH-T 13518	**I**	BAH (KM365926)	BAH	KM365639	-	KM365710
126	27	**30**	Ilhéus	BA	CFBH 4464	CFBH-T 171	**I**	BAH ([b]HQ262463.1)	BAH	[b]HQ262441.1	[b]HQ262425.1	KM365711
127	27	**414**	Ilhéus	BA	-	CFBH-T 13542	-	BAH (KM365927)	BAH	KM365640	KM365576	-
128	27	**415**	Ilhéus	BA	-	CFBH-T 13543	-	BAH (KM365928)	-	-	-	-
129	27	**416**	Ilhéus	BA	-	CFBH-T 13544	-	BAH (KM365929)	-	-	-	-
130	27	**417**	Ilhéus	BA	-	CFBH-T 13545	-	BAH (KM365930)	-	-	-	-
131	27	**418**	Ilhéus	BA	-	CFBH-T 13546	-	BAH (KM365931)	-	-	-	-
132	27	**419**	Ilhéus	BA	-	CFBH-T 13547	-	BAH (KM365932)	-	-	-	-
133	27	**420**	Ilhéus	BA	-	CFBH-T 13548	-	BAH (KM365933)	-	-	-	-
134	27	**421**	Ilhéus	BA	-	CFBH-T 13549	-	BAH (KM365934)	-	-	-	-
135	27	**422**	Ilhéus	BA	-	CFBH-T 13550	-	BAH (KM365935)	-	-	-	-
136	27	**423**	Ilhéus	BA	-	CFBH-T 13551	-	BAH (KM365936)	-	-	-	-
137	27	**424**	Ilhéus	BA	-	CFBH-T 13552	-	BAH (KM365937)	-	-	-	-
138	28	**190**	Camacan	BA	-	CFBH-T 12558	-	BAH ([b]HQ262464.1)	BAH	[b]HQ262442.1	[b]HQ262426.1	-
139	28	**510**	Camacan	BA	MHNJCH 248		**U**	BAH (KM365938)	-	-	-	-
140	28	**511**	Camacan	BA	MHNJCH 249		**S**	BAH (KM365939)	-	-	-	-
141	28	**526**	Camacan	BA	MHNJCH 356		**U**	BAH (KM365940)	-	-	-	-
142	28	**527**	Camacan	BA	MHNJCH 357		**I**	BAH (KM365941)	-	-	-	-
143	28	**528**	Camacan	BA	MHNJCH 358		**I**	BAH (KM365942)	-	-	-	-
144	28	**529**	Camacan	BA	MHNJCH 359		**I**	BAH (KM365943)	-	-	-	-
145	28	**530**	Camacan	BA	MHNJCH 360		**I**	BAH (KM365944)	-	-	-	-
146	28	**531**	Camacan	BA	MHNJCH 361		**I**	BAH (KM365945)	-	-	-	-
147	28	**537**	Camacan	BA	MHNJCH 388		**I**	BAH (KM365946)	-	-	-	-
148	28	**538**	Camacan	BA	MHNJCH 389		**I**	BAH (KM365947)	-	-	-	-
149	28	**539**	Camacan	BA	MHNJCH 390		**I**	BAH (KM365948)	-	-	-	-
150	28	**540**	Camacan	BA	MHNJCH 391		**I**	BAH(KM365949)	-	-	-	-
151	28	**541**	Camacan	BA	MHNJCH 392		**I**	BAH (KM365950)	-	-	-	-
152	29	**542**	Itapebi	BA	MHNJCH 393		**I**	BUR (KM365951)	-	-	-	-
153	29	**543**	Itapebi	BA	MHNJCH 394		**S**	BUR (KM365952)	-	-	-	-
154	29	**544**	Itapebi	BA	MHNJCH 395		**I**	BUR (KM365953)	-	-	-	-
155	29	**545**	Itapebi	BA	MHNJCH 396		**I**	BUR (KM365954)	-	-	-	-
156	29	**546**	Itapebi	BA	MHNJCH 397		**I**	BUR (KM365955)	-	-	-	-
157	30	**287**	Porto Seguro	BA	MNRJ 42660		**I**	BUR (KM365956)	BUR	-	-	KM365712
158	30	**288**	Porto Seguro	BA	MNRJ 42661		**I**	BUR (KM365957)	BUR	KM365641	-	KM365713
159	31	**547**	Itabela	BA	MHNJCH 398		**I**	BUR (KM365958)	-	-	-	-
160	31	**549**	Itabela	BA	MHNJCH 400		**I**	BUR (KM365959)	BAH/BUR	KM365642	-	KM365714
161	32	**225**	Caraíva	BA	CFBH 13353	CFBH-T 4124	**I**	BAH (KM365960)	-	-	-	-
162	33	**532**	Prado	BA	MHNJCH 371		**I**	BUR (KM365961)	-	-	-	-
163	33	**533**	Prado	BA	MHNJCH 372		**I**	BUR (KM365962)	-	-	-	-
164	33	**534**	Prado	BA	MHNJCH 373		**I**	BUR (KM365963)	-	-	-	-
165	33	**535**	Prado	BA	MHNJCH 374		**U**	BUR (KM365964)	BUR	KM365643	KM365577	KM365715
166	33	**536**	Prado	BA	MHNJCH 387		**I**	BUR (KM365965)	BAH/BUR	KM365644	-	KM365716
167	33	**548**	Prado	BA	MHNJCH 399		**I**	BUR (KM365966)	BAH/BUR	-	-	KM365717
168	34	**25**	Sooretama	ES	MNRJ 35007		**I**	BUR (KM365967)	BUR	-	-	KM365718
169	34	**116**	Sooretama	ES	CFBH 14908	CFBH-T 17886	**I**	BUR (KM365968)	-	-	-	-
170	35	**19**	Linhares	ES	MNRJ 22721	CFBH-T 152		BUR ([b]HQ262466.1)	BUR	[b]HQ262445.1	[b]HQ262429.1	[a]GQ366011.1

N	LC	Lab nº	Locality	ES	Voucher nº	Tissue nº	Morph.	ND2	STRUC.	C-myc2	β-fibin7	CXCR4
171	35	**26**	Linhares	ES	CFBH 11080	CFBH-T 4186	**I**	BUR (KM365969)	BUR	KM365645	-	KM365719
172	35	**193**	Linhares	ES	CFBH 18083	CFBH-T 9021	**I**	BUR (KM365970)	BUR	-	KM365578	KM365720
173	35	**194**	Linhares	ES	CFBH 18084	CFBH-T 9031	**I**	BUR (KM365971)	BUR	KM365646	-	KM365721
174	35	**286**	Linhares	ES	CFBH 24841	CFBH-T 11900	**I**	BUR (KM365972)	BUR	-	-	KM365722
175	36	**11**	Aracruz	ES	CFBH 2661	CFBH-T 90	**I**	BUR ([b]HQ262467.1)	BUR	[b]HQ262444.1	[b]HQ262428.1	KM365723
176	36	**12**	Aracruz	ES	CFBH 2662	CFBH-T 121	**I**	BUR (KM365973)	BUR	KM365647	KM365579	KM365724
177	36	**13**	Aracruz	ES	CFBH 2663	CFBH-T 122	**I**	BAH (KM365974)	BUR	-	KM365580	KM365725
178	36	**14**	Aracruz	ES	CFBH 5378	CFBH-T 123	**I**	BAH (KM365975)	BUR	KM365648	-	KM365726
179	36	**15**	Aracruz	ES	CFBH 5379	CFBH-T 124	**I**	BAH (KM365976)	BUR	KM365649	KM365581	KM365727
180	36	**16**	Aracruz	ES	CFBH 5380	CFBH-T 125	**I**	BAH (KM365977)	BUR	KM365650	-	KM365728
181	36	**17**	Aracruz	ES	CFBH 5381	CFBH-T 126	**I**	BUR (KM365978)	BUR	-	KM365582	KM365729
182	36	**18**	Aracruz	ES	CFBH 5382	CFBH-T 127	**I**	BUR (KM365979)	BUR	KM365651	-	KM365730
183	37	**24**	Santa Teresa	ES	MNRJ 34931		**I**	BUR (KM365980)	BUR	KM365652	KM365583	KM365731
184	37	**289**	Santa Teresa	ES	MNRJ 56012		**I**	BUR (KM365981)	BUR	-	-	KM365732
185	38	**568**	São João Evangelista	MG	UFMG-A 9588	UFMG-T 1715b	**S**	BUR (KM365982)	BUR	KM365653	-	KM365733
186	39	**157**	Catas Altas	MG	MNRJ 38482		**I**	BUR (KM365983)	-	-	-	KM365734
187	39	**158**	Catas Altas	MG	MNRJ 49667		**I**	BUR (KM365984)	BUR	-	-	KM365735
188	39	**159**	Catas Altas	MG	MNRJ 49668		**S**	BUR (KM365985)	BUR	-	-	KM365736
189	39	**564**	Catas Altas	MG	UFMG-A 1602	UFMG-T 595b	**I**	BUR (KM365986)	BUR	KM365654	-	KM365737
190	40	**563**	Congonhas	MG	UFMG-A 1606	UFMG-T 574b	**S**	BUR (KM365987)	BUR	KM365655	-	KM365738
191	41	**238**	Furnas	MG	CFBH 17360	CFBH-T 10423	-	BUR (KM365988)	-	-	-	[a]GQ366012.1
192	42	**361**	Viçosa	MG	CFBH 27579	CFBH-T 13325	**I**	BUR (KM365989)	-	-	-	KM365739
193	42	**362**	Viçosa	MG	CFBH 27580	CFBH-T 13326	**I**	BUR (KM365990)	BUR	KM365656	-	KM365740
194	42	**363**	Viçosa	MG	CFBH 27581	CFBH-T 13327	**I**	BUR (KM365991)	-	-	-	KM365741
195	42	**364**	Viçosa	MG	CFBH 27582	CFBH-T 13328	**S**	BUR (KM365992)	-	-	-	KM365742
196	42	**365**	Viçosa	MG	CFBH 27583	CFBH-T 13329	**I**	BUR (KM365993)	-	-	-	KM365743
197	42	**366**	Viçosa	MG	CFBH 27584	CFBH-T 13330	**I**	BUR (KM365994)	BUR	-	-	KM365744
198	42	**367**	Viçosa	MG	CFBH 27585	CFBH-T 13331	**I**	BUR (KM365995)	BUR	-	-	KM365745
199	43	**316**	Carangola	MG	CFBH 27295	CFBH-T 12948	**I**	BUR (KM365996)	-	-	-	KM365746
200	43	**318**	Carangola	MG	CFBH 27297	CFBH-T 12950	**S**	BUR (KM365997)	BUR	-	KM365584	KM365747
201	43	**319**	Carangola	MG	CFBH 27298	CFBH-T 12951	**S**	BUR (KM365998)	-	-	-	KM365748
202	43	**320**	Carangola	MG	CFBH 27299	CFBH-T 12952	**I**	BUR (KM365999)	BUR	KM365657	KM365585	KM365749
203	43	**321**	Carangola	MG	CFBH 27300	CFBH-T 12953	**I**	BUR (KM366000)	BUR	KM365658	-	KM365750
204	43	**322**	Carangola	MG	CFBH 27301	CFBH-T 12954	**I**	BUR (KM366001)	BUR	-	KM365586	KM365751
205	43	**323**	Carangola	MG	CFBH 27302	CFBH-T 12955	**I**	BUR (KM366002)	-	-	-	-
206	43	**324**	Carangola	MG	CFBH 27303	CFBH-T 12956	**I**	BUR (KM366003)	BUR	KM365659	-	KM365752
207	43	**325**	Carangola	MG	CFBH 27304	CFBH-T 12957	**S**	BUR (KM366004)	BUR	-	-	KM365753
208	43	**326**	Carangola	MG	CFBH 27305	CFBH-T 12958	**I**	BUR (KM366005)	BUR	-	KM365587	KM365754
209	43	**327**	Carangola	MG	CFBH 27315	CFBH-T 12968	**I**	BUR (KM366006)	-	-	-	KM365755
210	44	**310**	Juiz de Fora	MG	CFBH 27257	CFBH-T 12326	**I**	BUR (KM366007)	BUR	-	-	KM365756
211	44	**368**	Juiz de Fora	MG	CHUFJF 1002	CFBH-T-13364	**S**	BUR (KM366008)	-	-	-	KM365757
212	44	**369**	Juiz de Fora	MG	CHUFJF 1003	CFBH-T-13365	**I**	BUR (KM366009)	-	-	-	KM365758
213	44	**370**	Juiz de Fora	MG	CHUFJF 1004	CFBH-T-13366	**I**	BUR (KM366010)	BUR	KM365660	-	KM365759
214	44	**371**	Juiz de Fora	MG	CHUFJF 1005	CFBH-T-13367	**S**	BUR (KM366011)	BUR	KM365661	-	KM365760
215	44	**372**	Juiz de Fora	MG	CHUFJF 1006	CFBH-T-13368	**I**	BUR (KM366012)	BUR	-	-	KM365761
216	44	**373**	Juiz de Fora	MG	CHUFJF 1007	CFBH-T-13369	**S**	BUR (KM366013)	-	-	-	KM365762
217	44	**374**	Juiz de Fora	MG	CHUFJF 1008	CFBH-T-13370	**I**	BUR (KM366014)	BUR	KM365662	KM365588	KM365763
218	44	**375**	Juiz de Fora	MG	CHUFJF 1009	CFBH-T-13371	**S**	BUR (KM366015)	-	-	-	KM365764
219	44	**376**	Juiz de Fora	MG	CHUFJF 1010	CFBH-T-13372	**I**	BUR (KM366016)	-	-	-	KM365765
220	44	**377**	Juiz de Fora	MG	CHUFJF 1011	CFBH-T-13373	**I**	BUR (KM366017)	-	-	-	KM365766
221	44	**378**	Juiz de Fora	MG	CHUFJF 1012	CFBH-T-13374	**I**	BUR (KM366018)	-	-	-	KM365767
222	44	**379**	Juiz de Fora	MG	CHUFJF 1013	CFBH-T-13375	**I**	BUR (KM366019)	-	-	-	KM365768
223	45	**561**	Três Rios	RJ	MNRJ 78162		**S**	BUR (KM366020)	BUR	KM365663	KM365589	KM365769
224	46	**333***	Campos dos Goytacazes	RJ	CFBH 27386	CFBH-T 13045	**I**	BUR RJ (KM366021)	BUR	KM365664	KM365590	KM365770
225	46	**334***	Campos dos Goytacazes	RJ	CFBH 27387	CFBH-T 13046	**S**	BUR RJ (KM366022)	BUR	KM365665	KM365591	KM365771
226	46	**335**	Campos dos Goytacazes	RJ	CFBH 27388	CFBH-T 13047	**I**	BUR RJ (KM366023)	-	-	-	-
227	46	**336**	Campos dos Goytacazes	RJ	CFBH 27389	CFBH-T 13048	**I**	BUR RJ (KM366024)	BUR	KM365666	KM365592	KM365772
228	46	**337***	Campos dos Goytacazes	RJ	CFBH 27390	CFBH-T 13049	**S**	BUR RJ (KM366025)	BUR	KM365667	KM365593	KM365773
229	47	**328***	Santa Maria Madalena	RJ	CFBH 27326	CFBH-T 12980	**I**	BUR RJ (KM366026)	BUR	KM365668	KM365594	KM365774
230	47	**329***	Santa Maria Madalena	RJ	CFBH 27327	CFBH-T 12981	**S**	BUR RJ (KM366027)	BUR	KM365669	KM365595	KM365775

N	LC	Lab nº	Locality	ES	Voucher nº	Tissue nº	Morph.	ND2	STRUC.	C-myc2	β-fibin7	CXCR4
231	47	**330***	Santa Maria Madalena	RJ	CFBH 27328	CFBH-T 12982	**S**	BUR RJ (KM366028)	BUR	KM365670	KM365596	KM365776
232	47	**331**	Santa Maria Madalena	RJ	CFBH 27329	CFBH-T 12983	**I**	BUR RJ (KM366029)	BUR	-	KM365597	KM365777
233	48	**435***	Cachoeiras de Macacu	RJ	-	LAF 158	**-**	BUR RJ (KM366030)	BUR	KM365671	KM365598	KM365778
234	48	**440***	Cachoeiras de Macacu	RJ	-	LAF 159	**-**	BUR RJ (KM366031)	BUR	KM365672	KM365599	KM365779
235	48	**441**	Cachoeiras de Macacu	RJ	-	LAF 160	**-**	BUR RJ (KM366032)	BUR	-	**-**	KM365780
236	48	**466**	Cachoeiras de Macacu	RJ	CFBH 30764	CFBH-T 15085		BUR RJ (KM366033)	**-**	-	**-**	**-**
237	48	**467**	Cachoeiras de Macacu	RJ	CFBH 30766	CFBH-T 15087	**S**	BUR RJ (KM366034)	**-**	-	**-**	**-**
238	48	**468**	Cachoeiras de Macacu	RJ	CFBH 30767	CFBH-T 15088	**I**	BUR RJ (KM366035)	**-**	-	**-**	**-**
239	48	**469**	Cachoeiras de Macacu	RJ	CFBH 30768	CFBH-T 15089	**I**	BUR RJ (KM366036)	BUR	KM365673	**-**	KM365781
240	49	**290***	Niterói	RJ	MNRJ 51540		**S**	BUR RJ (KM366037)	BUR	KM365674	KM365600	KM365782
241	50	**560**	Rio de Janeiro	RJ	MNRJ 60678		**S**	BUR RJ (KM366038)	**-**	-	**-**	**-**
242	51	**23**	Queluz	SP	CFBH 7204	CFBH-T 877	**S**	BUR (KM366039)	**-**	-	**-**	KM365783
243	52	**177***	São José do Rio Pardo	SP	CFBH 18657	CFBH-T 8121	**I**	BUR (KM366040)	BUR	KM365675	KM365601	KM365784
244	52	**178***	São José do Rio Pardo	SP	CFBH 18658	CFBH-T 8122	**I**	BUR (KM366041	BUR	KM365676	KM365602	KM365785
245	52	**179**	São José do Rio Pardo	SP	CFBH 18659	CFBH-T 8123	**S**	BUR (KM366042)	BUR	-	KM365603	KM365786
246	52	**180**	São José do Rio Pardo	SP	CFBH 18660	CFBH-T 8124	**I**	BUR (KM366043	BUR	-	KM365604	KM365787
247	52	**181**	São José do Rio Pardo	SP	CFBH 18661	CFBH-T 8125	**I**	BUR (KM366044	**-**	-	**-**	KM365788
248	53	**118***	Rio Claro	SP	CFBH 14428	CFBH-T 5310	**I**	BUR ([b]HQ262468.1)	BUR	KM365677	KM365605	KM365789
249	54	**22***	Jundiaí	SP	CFBH 2053	CFBH-T 179	**I**	BUR ([b]HQ262469.1)	BUR	[b]HQ262446.1	[b]HQ262430.1	KM365790
250	54	**281***	Jundiaí	SP	CFBH 24830	CFBH-T 11893	**-**	BUR (KM366045)	BUR	-	KM365606	KM365791
251	54	**282***	Jundiaí	SP	CFBH 24831	CFBH-T 11894	**-**	BUR (KM366046)	**-**	-	KM365607	KM365792
252	54	**283***	Jundiaí	SP	CFBH 24832	CFBH-T 11895	**-**	BUR (KM366047)	**-**	-	KM365608	KM365793
253	54	**284***	Jundiaí	SP	CFBH 24833	CFBH-T 11896	**-**	BUR (KM366048)	**-**	-	KM365609	KM365794
254	54	**332**	Jundiaí	SP	CFBH 27449	CFBH-T 12998	**I**	BUR (KM366049)	**-**	-	**-**	KM365795
255	54	**462**	Jundiaí	SP	CFBH 29857	CFBH-T 14642	**I**	BUR (KM366050)	**-**	-	**-**	**-**
256	54	**463**	Jundiaí	SP	CFBH 29858	CFBH-T 14643	**I**	BUR (KM366051)	**-**	-	**-**	KM365796
257	54	**464**	Jundiaí	SP	CFBH 29859	CFBH-T 14644	**I**	BUR (KM366052)	**-**	-	**-**	KM365797
258	54	**465**	Jundiaí	SP	CFBH 29860	CFBH-T 14645	**I**	BUR (KM366053)	**-**	-	**-**	**-**
259	54	**509**	Jundiaí	SP	CFBH 31061	CFBH-T 15283	**I**	BUR (KM366054)	BUR	-	KM365610	KM365798
260	55	**470**	Nazaré Paulista	SP	CFBH 30573	CFBH-T 15180	**S**	BUR (KM366055)	**-**	-	**-**	**-**
261	55	**471**	Nazaré Paulista	SP	CFBH 30574	CFBH-T 15181	**I**	BUR (KM366056)	**-**	-	**-**	**-**
262	55	**472**	Nazaré Paulista	SP	CFBH 30575	CFBH-T 15182	**S**	BUR (KM366057)	**-**	-	**-**	**-**
263	56	**128**	São Paulo	SP	-	MTR-AF 145	**-**	BUR (KM366058)	BUR	-	KM365611	KM365799
264	56	**146***	São Bernardo	SP	IT-H0136	MTR-AF 1068	**-**	BUR (KM366059)	BUR	KM365678	KM365612	KM365800
265	56	**147**	São Bernardo	SP	IT-H0119	MTR-AF 1063	**-**	BUR (KM366060)	BUR	KM365679	KM365613	KM365801
266	56	**192**	Caieiras	SP	CFBH 19696	CFBH-T 9029	**I**	BUR (KM366061)	BUR	KM365680	**-**	KM365802
267	57	**129**	Iguape	SP	MTR 09562	MTR-AF 146	**-**	BUR (KM366062)	BUR	-	KM365614	KM365803
268	57	**130**	Iguape	SP	MTR 09557	MTR-AF 150	**-**	BUR (KM366063)	**-**	-	**-**	**-**
269	57	**134**	Iguape	SP	MTR 09560	MTR-AF 173	**-**	BUR (KM366064)	**-**	-	**-**	**-**
270	-	**28**	Iporanga	SP	CFBH 13868	CFBH-T 4295	**-**	*P. distincta* [b]HQ262480.1	**-**	[b]HQ262450.1	[b]HQ262434.1	KM365804
271	-	**32**	Guaratuba	PR	CFBH 2658	CFBH-T 120	**-**	*P. distincta* [b]HQ262482.1	**-**	[b]HQ262448.1	[b]HQ262432.1	KM365805
272	-	**48**	Treviso	SC	CFBH 10321	CFBH-T 2407	**-**	*P. distincta* (KM366065)	**-**	[b]HQ262447.1	[b]HQ262431.1	-
273	-	**51**	Barra Velha	SC	CFBH 10999	CFBH-T 3023	**-**	*P. distincta* (KM366066)	**-**	[b]HQ262449.1	[b]HQ262433.1	-
274	-	**350**	Blumenau	SC	CFBH 27519	CFBH-T 13175	**-**	*P. distincta* (-)	**-**	-	-	KM365806
275	-	**357**	Treviso	SC	CFBH 27561	CFBH-T 13217	**-**	*P. distincta* (-)	**-**	-	-	KM365807
276	-	**3**	Santa Maria	RS	CFBH 3894	CFBH-T 182	**-**	*P. iheringii* [b]HQ262488.1	**-**	[b]HQ262454.1	[b]HQ262438.1	KM365808
277	-	**4**	Santa Maria	RS	MNRJ 18782	CFBH-T 183	**-**	*P. iheringii* [b]HQ262489.1	**-**	[b]HQ262455.1	[b]HQ262439.1	KM365809
278	-	**152**	Corumbá	MS	CFBH 2570	CFBH-T 173	**-**	*P. boliviana* [b]HQ262456.1	**-**	[b]HQ262440.1	[b]HQ262424.1	KM365810

[a] Faivovich *et al.* (2009)
[b] Brunes *et al.* (2010)
* Allopatric dataset
- no information or no voucher
CFBH; Célio Fernando Baptista Haddad specimen collection
CFBH-T; Célio Fernando Baptista Haddad - Tissue collection
MNRJ; Museu Nacional da Universidade Federal do Rio de Janeiro
MHNJCH; Museu de História Natural de Jequié Coleção Herpetológica
UFBA; Universidade Federal da Bahia specimen collection
UFBA-T; Universidade Federal da Bahia - Tissue collection
UFMG-A; Universidade Federal de Minas Gerais - Specimen collection
UFMG-T; Universidade Federal de Minas Gerais - Tissue collection
CHUFJF; Coleção Herpetológica da Universidade Federal de Juiz de Fora specimen collection
LAF; Luciana Ardenghi Fusinatto field numbers
IT-H and MTR; Miguel Trefaut Rodrigues field numbers
MTR-AF; Miguel Trefaut Rodrigues lab numbers

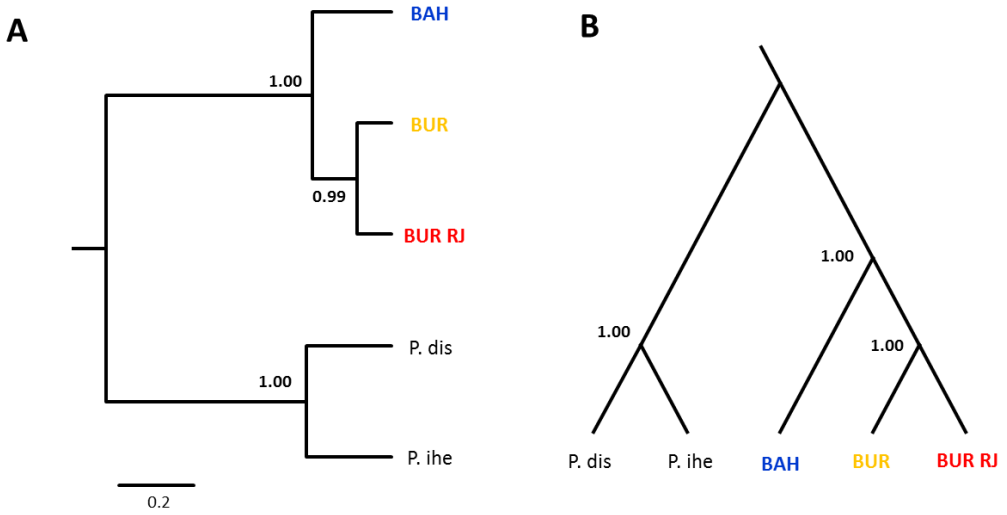

Fig. B.1 Multilocus nuclear analysis: (A) species tree in *BEAST and (B) species delimitation in BPP. Posterior probabilities are shown near the branches. P. dis, *Phyllomedusa distincta*; P. ihe, *Phyllomedusa iheringii*.

Table B.2 Comparisons of pairwise FST values above the diagonal and pairwise values of Da (Nei *et al.* 1983) genetic distance for the 10 populations/localities (see Fig. 3.7 for codes) of *Phyllomedusa burmeisteri* with n ≥ 5. ns, not significant.

Pop/Loc	4	6	7	13	14	15	17	19	22	25
4	-	0.019 ns	0.073	0.057	0.025	0.050	0.119	0.101	0.114	0.054
6	0.606	-	0.066	0.066	0.051	0.057	0.122	0.075	0.139	0.084
7	0.694	0.644	-	0.096	0.072	0.104	0.165	0.130	0.176	0.107
13	0.640	0.664	0.683	-	0.070	0.076	0.159	0.116	0.154	0.121
14	0.518	0.648	0.588	0.629	-	0.058	0.110	0.096	0.132	0.079
15	0.653	0.668	0.721	0.638	0.590	-	0.122	0.106	0.155	0.082
17	0.723	0.723	0.772	0.758	0.658	0.616	-	0.116	0.246	0.173
19	0.752	0.642	0.713	0.682	0.643	0.648	0.645	-	0.190	0.164
22	0.664	0.738	0.775	0.661	0.644	0.708	0.800	0.737	-	0.091
25	0.589	0.633	0.621	0.713	0.558	0.617	0.708	0.791	0.501	-

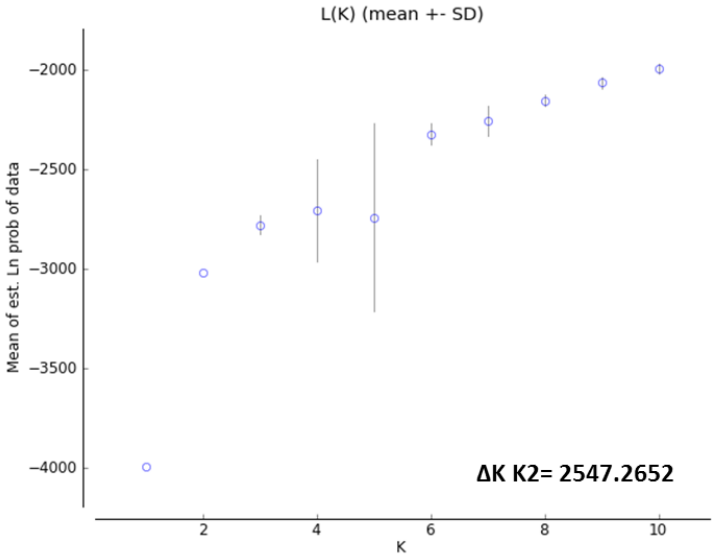

Fig. B.2 K values curve for K=1-10 (Ln Pr (X/K)), and value of ΔK for K=2.

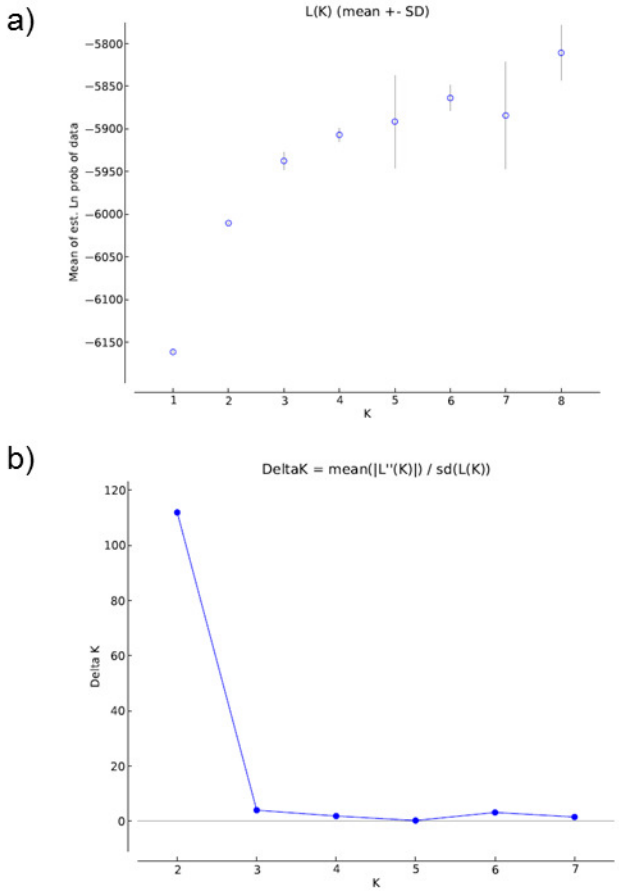

Fig. B.3 Inference of K (the most probable number of clusters) using STRUCTURE software based on microsatellite analysis of 103 total individuals of *Phyllomedusa burmeisteri* (a) Log-likelihood value of data L(K) as a function of K averaged over five replicates (b) Second order of change of the log-likelihood of the data (Δ K) as a function of K, calculated over five replicates.

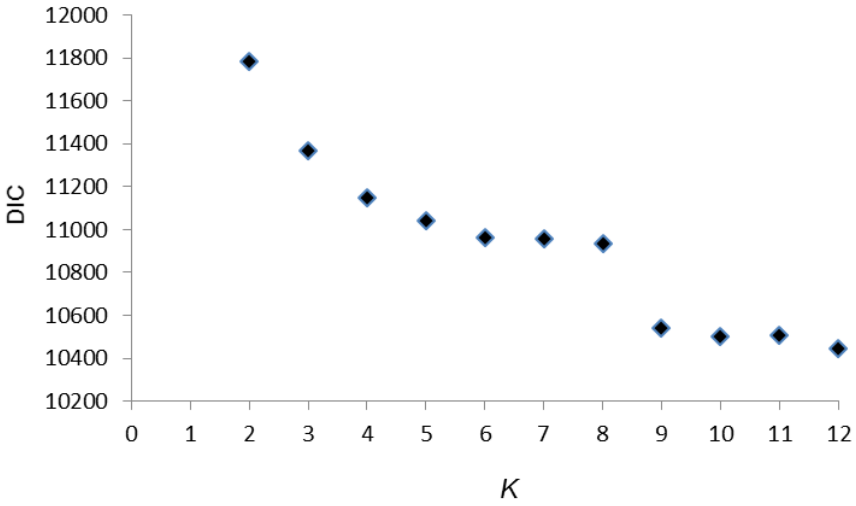

Fig. B.4 Determination of the number of clusters in the microsatellite data set of *Phyllomedusa burmeisteri* using the deviance information criterion (DIC) of results from the TESS (Chen *et al*., 2007) obtained under the admixture model with interaction parameter ψ=0.7 and a linear trend degree surface. DIC close to the asymptote indicates the best K, suggesting here that K=4 best explains the population structure.

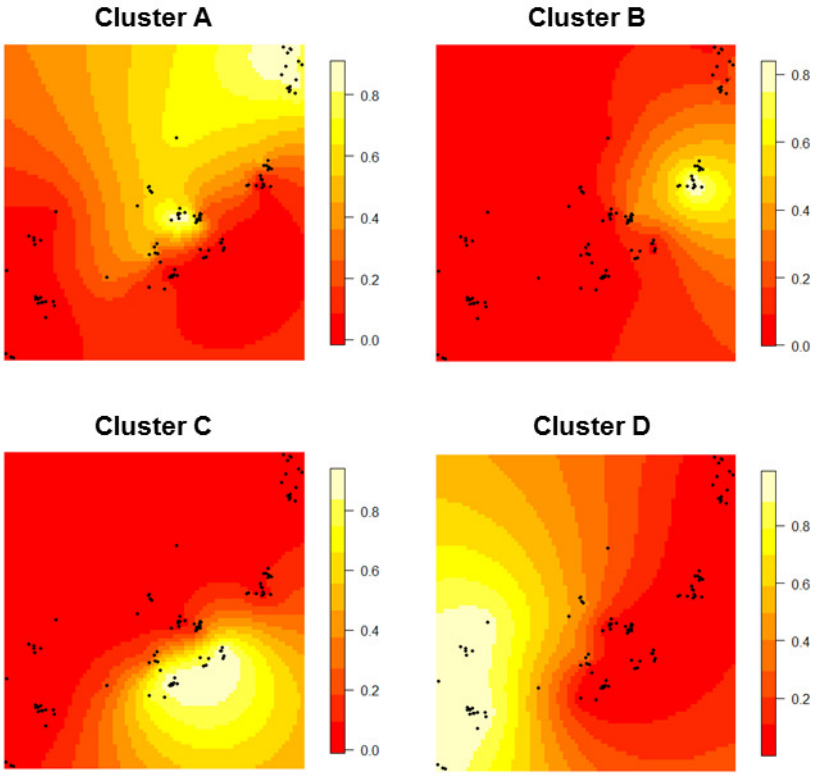

Fig. B.5 Spatially explicit estimate of population structure of *Phyllomedusa burmeisteri* based on the TESS results for K=4. Results were obtained across 5 runs using CLUMPP (Jakobsen & Rosenberg 2007) and displayed through the spatial interpolation method (Kriging) implemented in TESS. Each of K=4 clusters is displayed in a separate graph, in which the highest posterior probabilities are in white and the lowest are in red. Black dots represent each individual.

Anexo C

Material suplementar para a Secção 4.1

Table C.1 Species-specific tuning results for *Phyllomedusa distincta* carried on with 10 replicated runs under the current climatic conditions in Maxent. Bold type indicates the selected regularization parameter. See text for abbreviations details.

Regularization parameter	Average test AUC for the replicate (SD)	AUC differences (training – test)	MTP test omission rates	Variable response curves
0.25	0.912 (0.032)	0.04	0.10	Irregular
0.5	0.92 (0.027)	0.03	0.17	Regular, less smooth
1.0 (default)	0.912 (0.03)	0.03	0.07	Regular & smooth
1.5	0.93 (0.023)	0.01	0.03	Regular & smooth
2.0	0.917 (0.027)	0.025	0.1	Regular, less smooth
4.0	0.927 (0.023)	-0.003	0.07	Regular, less smooth
6.0	0.908 (0.028)	-0.003	0.01	More flat
8.0	0.983 (0.040)	-0.013	0.04	More flat
10	0.886 (0.040)	-0.019	0.04	More flat

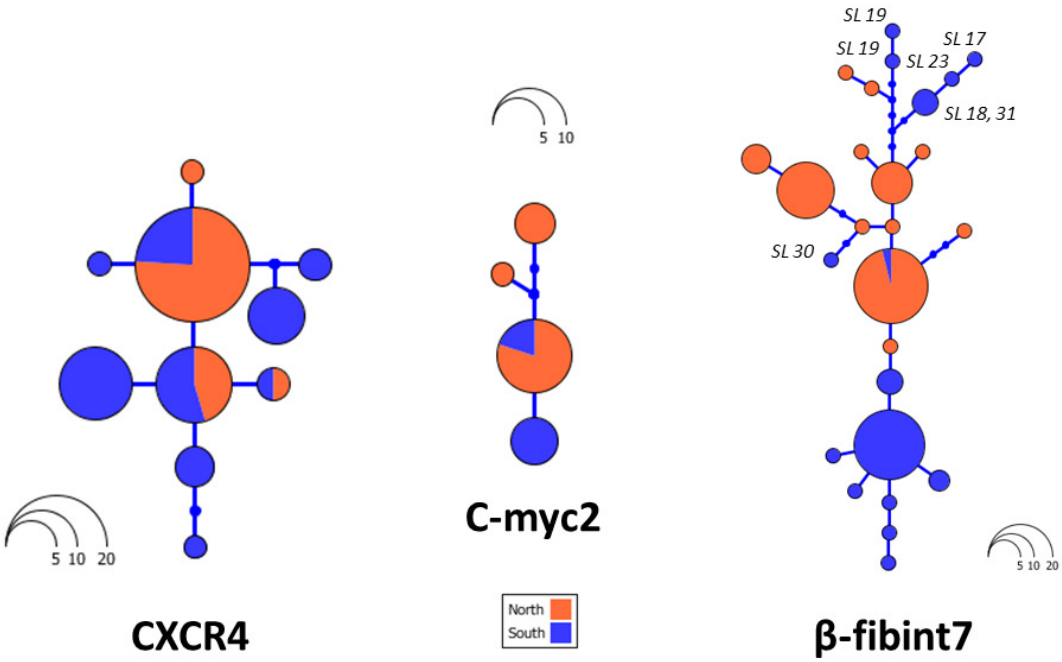

Fig. C.1 Nuclear haplotype genealogies of *Phyllomedusa distincta* converted from parsimony tree in the software Haploviewer. The circle area of each haplotype is proportional to its frequency. Mutations are edges. Colors represents the two major mitochondrial groups (see Fig. 4.1). Numbers near haplotypes indicate sampling localities, SL (see Table 4.1 and Fig. 4.1).

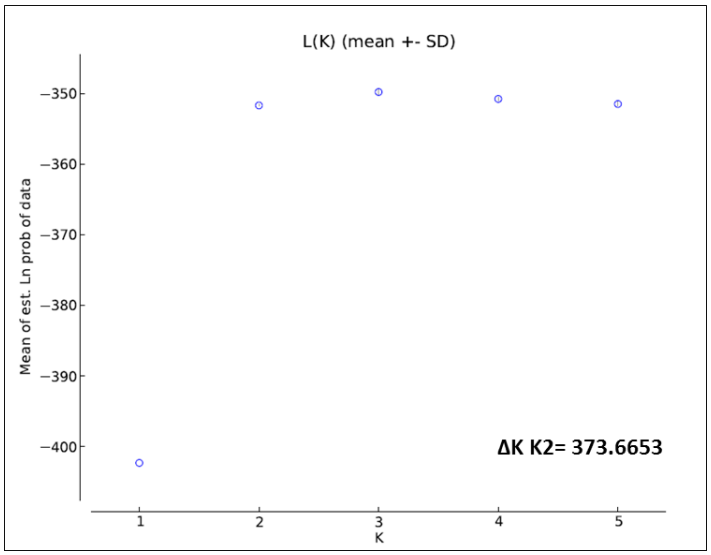

Fig. C.2 K values curve for K=1-10 (Ln Pr (X/K)), and value of ΔK for K=2.

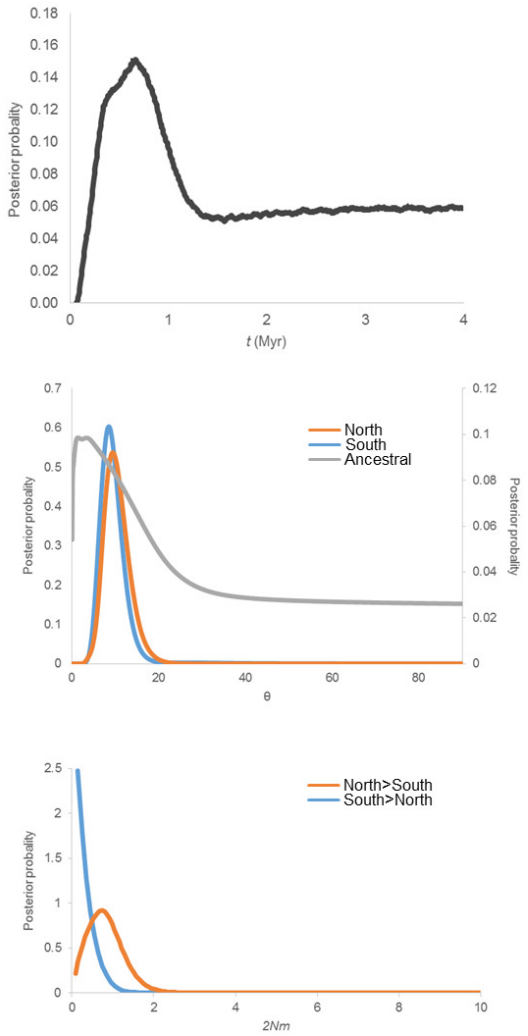

Fig. C.3 Marginal posterior probability densities for divergence time (t), theta (θ) and population migration rates (2Nm) estimates using Isolation-with-Migration model (IMa2) for five pairwise comparisons.

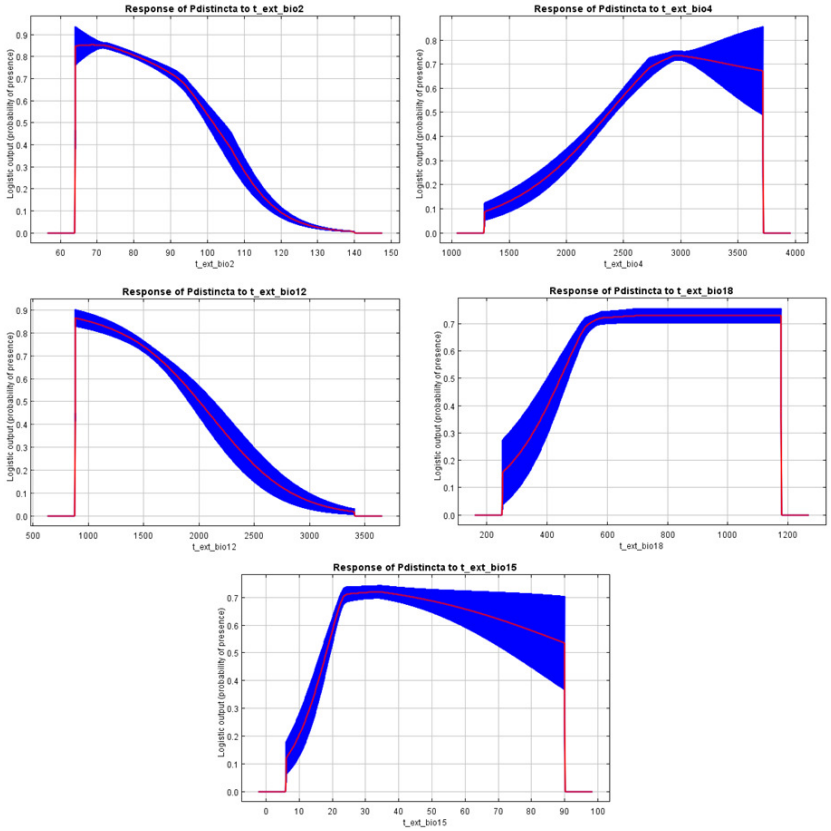

Fig. C.4 Response curves of the 10 replicate Maxent runs (red) and standard deviation (blue) of *Phyllomedusa distincta* models using the selected regularization parameter (1.0). The curves show how each environmental variable affects the Maxent prediction.

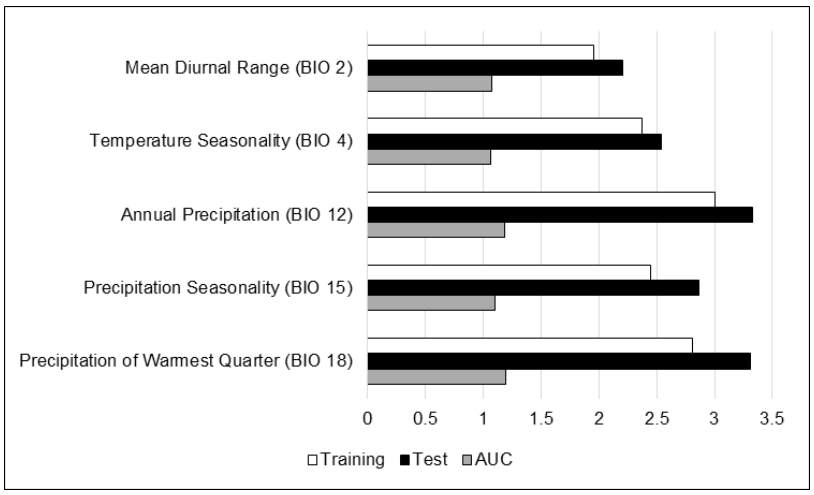

Fig. C.5 Jackknife results for 10 Maximum Entropy models of the distribution of *Phyllomedusa distincta* in the Brazilian Atlantic forest. To evaluate de importance of each variable in the model's constructions, the difference in average gain and area under the curve (AUC) on test data between models built without a given variable and models built with only that variable ((without – with only) + 1). Variables with smaller differences both in gain and in AUC are the most related to the distribution of species.

Anexo D

Material suplementar para a Secção 5.1

Table D.1 Occurrence points information of *Phyllomdusa distincta*, *Phyllomedusa tetraploidea*, and *Phyllomedusa iheringii* used in GIS-based analysis.

Species	Long	Lat	Locality	State	Country	Collection database or reference
P. distincta	-46.8356	-23.5225	Carapicuiba	SP	BRA	MTR/USP
P. distincta	-46.6279	-23.5752	São Paulo	SP	BRA	MTR/USP
P. distincta	-47.7164	-23.8131	Pilar do Sul	SP	BRA	CFBH/UNESP
P. distincta	-47.6785	-24.3280	Juquiá	SP	BRA	CFBH/UNESP
P. distincta	-48.0804	-24.5337	Eldorado	SP	BRA	CFBH/UNESP
P. distincta	-47.8811	-24.7150	Pariquera-Açu	SP	BRA	CFBH/UNESP
P. distincta	-47.5414	-24.6981	Iguape	SP	BRA	MTR/USP
P. distincta	-48.7003	-24.5328	Iporanga/Apiaí	SP	BRA	CFBH/UNESP
P. distincta	-48.4233	-24.1951	Ribeirão Grande	SP	BRA	CFBH/UNESP
P. distincta	-48.7430	-24.3586	Ribeirão Branco	SP	BRA	CFBH/UNESP
P. distincta	-49.0315	-24.6404	Adrianopolis	PR	BRA	CFBH/UNESP
P. distincta	-49.0444	-25.4422	Piraquara	PR	BRA	CFBH/UNESP
P. distincta	-48.7119	-25.4286	Antonina	PR	BRA	CFBH/UNESP
P. distincta	-48.5747	-25.8828	Guaratuba	PR	BRA	CFBH/UNESP
P. distincta	-48.8887	-25.9807	Garuva	SC	BRA	CFBH/UNESP
P. distincta	-49.3786	-26.2503	São Bento do Sul	SC	BRA	CFBH/UNESP
P. distincta	-49.0032	-26.5099	Guaramirim	SC	BRA	CFBH/UNESP
P. distincta	-49.2594	-26.8075	Timbó	SC	BRA	CFBH/UNESP
P. distincta	-49.0911	-27.0289	Blumenau	SC	BRA	CFBH/UNESP
P. distincta	-49.4142	-27.0980	Apiúna	SC	BRA	UFSC
P. distincta	-49.3304	-27.3604	Vidal Ramos	SC	BRA	EMLG/Unochapecó
P. distincta	-48.6918	-26.7242	Barra Velha	SC	BRA	CFBH/UNESP
P. distincta	-48.6537	-26.8330	Santa Lídia	SC	BRA	CFBH/UNESP
P. distincta	-48.5168	-27.1238	Porto Belo	SC	BRA	CFBH/UNESP
P. distincta	-48.5069	-27.4585	Florianópolis 1	SC	BRA	CFBH/UNESP
P. distincta	-48.5101	-27.5966	Florianópolis 2	SC	BRA	UFSC
P. distincta	-48.5438	-27.7307	Florianópolis 3	SC	BRA	UFSC
P. distincta	-49.0049	-27.5885	Angelina	SC	BRA	CFBH/UNESP
P. distincta	-48.8998	-28.1880	São Martinho	SC	BRA	CFBH/UNESP
P. distincta	-48.8536	-28.3111	Imbituba	SC	BRA	CFBH/UNESP
P. distincta	-49.4575	-28.5156	Treviso	SC	BRA	CFBH/UNESP
P. distincta	-49.8928	-29.3806	Dom Pedro de Alcântara	RS	BRA	CFBH/UNESP
P. tetraploidea	-49.9829	-22.1911	Marília	SP	BRA	CFBH/UNESP
P. tetraploidea	-50.3933	-22.5992	Assis	SP	BRA	CFBH/UNESP
P. tetraploidea	-48.5928	-23.7975	Buri	SP	BRA	CFBH/UNESP
P. tetraploidea	-48.4233	-24.1951	Ribeirão Grande	SP	BRA	CFBH/UNESP
P. tetraploidea	-48.7430	-24.3586	Ribeirão Branco	SP	BRA	CFBH/UNESP
P. tetraploidea	-49.6500	-24.1666	Jaguariaíva	PR	BRA	CFBH/UNESP

Species	Long	Lat	Locality	State	Country	Collection database or reference
P. tetraploidea	-50.7411	-23.7275	São Jerônimo da Serra	PR	BRA	CFBH/UNESP
P. tetraploidea	-50.9494	-24.2083	Ortigueira	PR	BRA	CFBH/UNESP
P. tetraploidea	-50.4635	-24.5354	Tibagi	PR	BRA	CFBH/UNESP
P. tetraploidea	-52.0101	-25.6975	Candói	PR	BRA	CFBH/UNESP
P. tetraploidea	-51.2583	-26.0784	Cruz Machado	PR	BRA	CFBH/UNESP
P. tetraploidea	-52.4550	-26.6314	São Domingos	SC	BRA	CFBH/UNESP
P. tetraploidea	-52.2639	-26.7595	Faxinal dos Guedes	SC	BRA	EMLG/Unochapecó
P. tetraploidea	-51.8115	-26.8270	Ponte Serrada	SC	BRA	EMLG/Unochapecó
P. tetraploidea	-51.7719	-27.4197	Piratuba	SC	BRA	CFBH/UNESP
P. tetraploidea	-52.9666	-27.1666	Caxambu do Sul	SC	BRA	EMLG/Unochapecó
P. tetraploidea	-53.3968	-26.7508	São Miguel do Oeste	SC	BRA	EMLG/Unochapecó
P. tetraploidea	-53.8502	-27.2298	Derrubadas	RS	BRA	EMLG/Unochapecó
P. tetraploidea	-54.4788	-26.9716	Misiones	Misiones	ARG	CONICET
P. tetraploidea	-55.6750	-26.5753	Alto Verá	Itapúa	PAR	IIBP/USP
P. iheringii	-53.8069	-29.6842	Santa Maria	RS	BRA	CFBH/UNESP
P. iheringii	-53.5653	-30.1606	São Sepé	RS	BRA	CFBH/UNESP
P. iheringii	-53.6725	-31.5581	Candiota	RS	BRA	MCT/PUCRS
P. iheringii	-54.2680	-31.3400	Bagé	RS	BRA	Pombal & Haddad (1992)
P. iheringii	-53.6690	-30.7120	Caçapava do Sul	RS	BRA	Pombal & Haddad (1992)
P. iheringii	-50.8680	-29.9610	Gravataí	RS	BRA	Pombal & Haddad (1992)
P. iheringii	-52.0440	-31.4130	São Lourenço do Sul	RS	BRA	Pombal & Haddad (1992)
P. iheringii	-53.8025	-32.1475	Rincón de Paiva	Cerro Largo	URU	FCIEN/ZVCB
P. iheringii	-55.0523	-34.3714	Salto del Penitente	Lavalleja	URU	FCIEN/ZVCB
P. iheringii	-54.9500	-33.5333	Zapicán	Lavalleja	URU	Nuñez *et al*. (2004)
P. iheringii	-53.7833	-32.7166	Placido Rosas	Cerro Largo	URU	Nuñez *et al*. (2004)
P. iheringii	-55.4333	-33.4500	Durazno	Durazno	URU	Nuñez *et al*. (2004)
P. iheringii	-55.2500	-34.9000	Punta Negra	Maldonado	URU	Nuñez *et al*. (2004)
P. iheringii	-55.3300	-31.2000	Establecimiento Trinidad	Rivera	URU	Nuñez *et al*. (2004)
P. iheringii	-55.9833	-31.1166	Puntas del Arroyo Lunarejo	Rivera	URU	Nuñez *et al*. (2004)
P. iheringii	-56.8833	-34.0666	Sierra de Mahoma	San Jose	URU	Nuñez *et al*. (2004)
P. iheringii	-54.4000	-32.9666	Quebrada de Los Cuervos	Treinta y Tres	URU	Nuñez *et al*. (2004)
P. iheringii	-55.0500	-32.9166	Santa Clara de Olimar	Treinta y Tres	URU	Nuñez *et al*. (2004)

CFBH/UNESP; Célio Fernando Baptista Haddad specimen collection, Brazil.

EMLG/Unochapecó; Elaine M. Lucas field numbers, Universidade Comunitária da Região de Chapecó, Brazil.
UFSC, Universidade Federal de Santa Catarina specimen collection, Brazil.
CONICET; Consejo Nacional de Investigaciones Científicas y Técnicas specimen collection, Argentine.
MTR/USP and IIBP; Miguel Trefaut Rodrigues lab and field numbers, Brazil.
MCT/PUCRS; Museu de Ciências e Tecnologia da Pontifícia Universidade Católica do Rio Grande do Sul specimen collection, Brazil.
FCIEN/ZVCB; Colección de Anfibios, Zoología Vertebrados, Facultad de Ciencias, Universidad de la República, Uruguay.

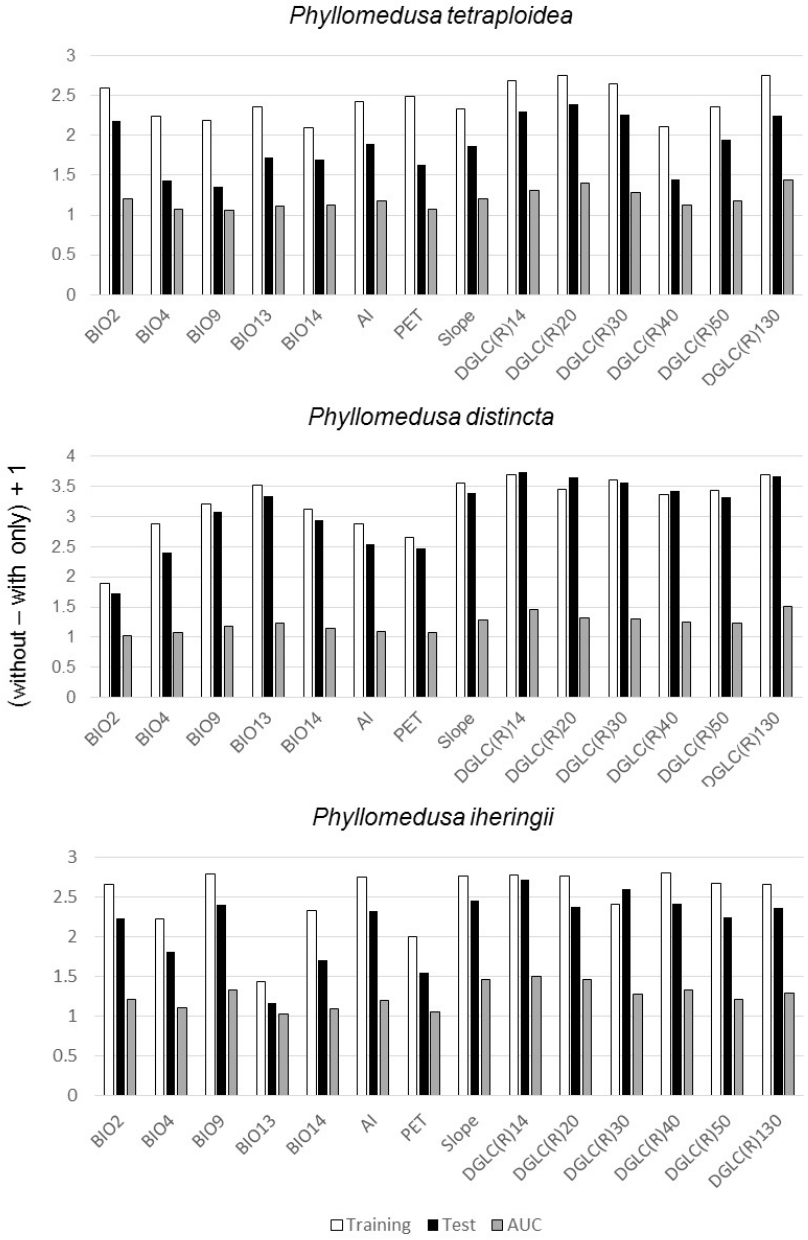

Fig. D.1 Jackknife results for 10 Maximum Entropy models of the distribution of *Phyllomedusa distincta*, *Phyllomedusa tetraploidea* and *Phyllomedusa iheringii* in the Atlantic and Pampeam forest. To evaluate de importance of each variable in the model's constructions, the difference in average gain and area under the curve (AUC) on test data between models built without a given variable and models built with only that variable ((without – with only) + 1).

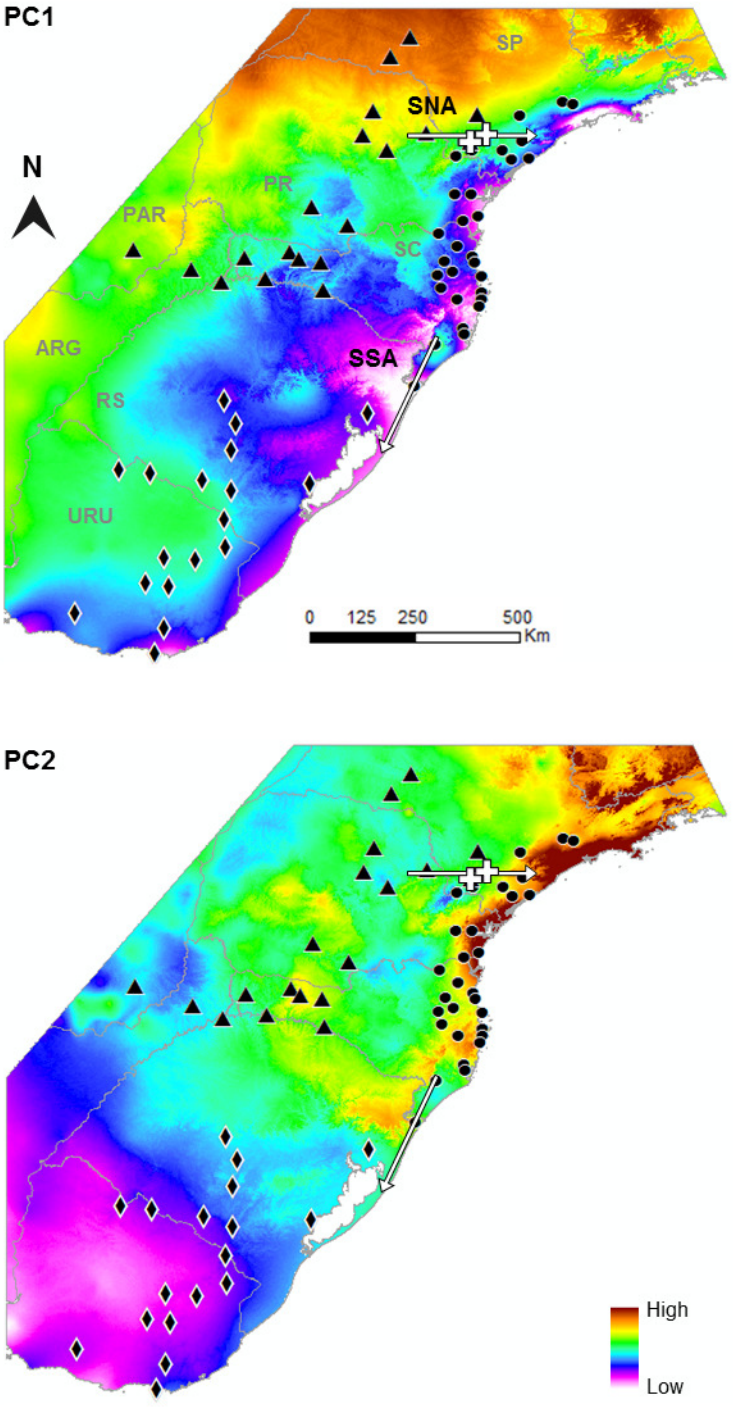

Fig. D.2 Spatial principal component maps for the first (PC1) and second (PC2) most informative axes. Analysis was performed with the six environmental variables most related with the potential occurrence of *Phyllomedusa tetraploidea* (triangles), *P. iheringii* (diamonds) and *P. distincta* (circles) (see Fig. D.1). White crosses represent the two described areas where *P. tetraploidea* and *P. distincta* hybridize (see text for details). White arrows indicate the position/direction of the analysed transects in the Sympatry North Area (SNA) and in the Sympatry South Area (SSA) (see Figs. 5.1 and 5.5). Acronyms indicate Brazilian states: SP, São Paulo; PR, Paraná; SC, Santa Catarina; and RS, Rio Grande do Sul; and countries: PAR, Paraguay; ARG, Argentine; and URU, Uruguay.